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Begin the article with a short summary.

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THIRD CONFERENCE ON BRAIN TUMOR THERAPY

Asilomar Conference Grounds
Pacific Grove, California
August 29-31, 1979

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Neil Buckely

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Opening Remarks to the Third Conference on Brain Tumor Therapy

Charles B. Wilson[1]

In preparing for this conference, from my own perspective I reviewed progress in the treatment of malignant gliomas over the past decade.

Operative mortality has reached an all-time steady low as a direct effect of perioperative use of glucocorticoids to reduce brain edema. A possible oncolytic effect of glucocorticoids is under investigation, but present evidence suggests that if there is an oncolytic effect it is small.

Radiation has assumed an unequivocal role in the treatment of malignant gliomas, and it is now clear that doses up to 6000 rads extend survival beyond that obtained with treatment at significantly lower levels of irradiation. Use of preoperative irradiation is being discussed, but even though clinical trials as a model for institutional groups have been completed in this country and abroad, the matter will not be resolved soon because of the difficulties in study design and agreement among neurosurgeons as to the extent of surgical resection.

The wide variety of animal models available for research on the efficacy of new therapeutic modalities has reached an historical maximum. Finally, chemotherapy has become recognized as an effective modality, and at least three drugs—BCNU, CCNU, and procarbazine—have shown significant activity in phase II trials.

This review of progress suggests a short list of things that are urgently needed. Perhaps most of all we need new antineoplastic drugs. In addition, we need a clearer and deeper understanding of pharmacokinetics in relation to drug delivery to tumors. We must gain insight into the true heterogeneity of malignant gliomas, and clonal drug resistance must be defined. The development of the necessary new agents will require new insights into glial cell function and differentiation. These cells must have a uniqueness that can and should be exploited.

In an attempt to anticipate the direction of laboratory and clinical research during the next several years, I place exploitation of glioma uniqueness at cellular and subcellular levels as the most important matter at hand. We should explore new models, especially those that involve human tumors. The use of radiosensitizers has a particular application to malignant gliomas, and undoubtedly these sensitizers can be used to effect a greater initial log cell kill. We will be revising our former concepts and considering better methods of drug delivery to tumor cells based on new knowledge of vascular anatomy and physiology in critical areas of a tumor. Investigators in our own group will be reconsidering interstitial chemotherapy and radiation therapy, and with others, we will intensify our serach for specific and nonspecific biochemical tumor markers that can be measured in blood and cerebrospinal fluid.

Finally, I see the primary emphasis of this conference placed jointly on the pharmacokinetics of drug delivery to CNS tumors and the heterogeneity of tumors as it relates to growth characteristics in vivo and in vitro and to drug sensitivity. The program is exciting and the speakers are known for their provocative statements and new approaches. I anticipate an informative conference, and I hope the relaxed atmosphere created by the organizers will be conducive to a free interchange of ideas.

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Regional Blood Flow, Capillary Permeability, and Glucose Utilization in Two Brain Tumor Models: Preliminary Observations and Pharmacokinetic Implications.

Ronald G. Blasberg,[1,*] Tomowo Kobayashi,[1] Clifford S. Patlak,[2] Mami Shinohara,[2] Makoto Miyoaka,[2] Jerry M. Rice,[1] and William R. Shapiro[3,4]

SUMMARY

Regional quantitative autoradiographic measurements of blood flow, capillary permeability, and glucose utilization were made in rats harboring an intracerebral Walker 256 metastatic tumor or a well-differentiated oligodendroglioma. Distinct differences were observed between the two brain tumor models; regional differences within the metastatic tumor appeared to be partly related to tumor size. The pharmacokinetic implications of these physiological measurements with respect to drug delivery to tumor tissue are discussed. The interrelationship of commonly used parameters to quantitate transport across the blood-brain barrier—permeability coefficient, permeability-surface area product, transfer constant, extraction fraction, equilibrium half-time, and blood flow—and their relevance to effective brain tumor chemotherapy are demonstrated.

[Cancer Treat Rep 65 (Suppl 2):3–12, 1981]

The transfer drugs or other substances between blood and tissue depends on the rate of capillary blood flow, the permeability and surface area of the capillaries, and the functional size of the tissue distribution spaces. Pathological conditions, such as a brain tumor, could significantly alter these physiological parameters as well as effect the energy metabolism of surrounding tissue. A relatively new technique for substrate measurements, quantitative autoradiography (QAR), has been developed by Sokoloff and co-workers (1–6) and applied to studies of regional cerebral blood flow (1–3), local cerebral glucose utilization (4–6), and blood to brain transport (7,8) in normal animals. Quantitative measurements of ^{14}C-radioactivity can be made in very small tissue regions ($100\mu = 100\mu \times 20\mu$) representing the volume of approximately 100–200 cells. Histological features such as tumor type, size, location, cell density, and necrosis can be directly compared with QAR measurements and analyzed in various ways using computer and imaging-assisted techniques.

This report presents preliminary results from a series of QAR studies in two experimental brain tumor models (9–11) and relates these findings to the more general problem of drug delivery to the tumor site. Abstracts of some aspects of this work have been presented (12,13). This presentation should be read in conjunction with those of Drs. Fenstermacher (14) and Levin (15) who discuss specific aspects of drug delivery to a particular tissue in greater detail using different pharmacokinetic models. The former presentation focuses on systemic parameters of drug delivery such as the site and rate of administration, the effects of distribution to the body mass, and the rate of plasma clearance; the magnitude of drug exposure or concentration-time product ($C \times T$) that can be achieved at

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[4]We thank Dr. Louis Sokoloff, Mrs. Jane Jehle, Mr. Charles Goochee, and Mr. James D. Brown of the Laboratory of Cerebral Metabolism, National Institute of Mental Health, for their generous support and assistance with the quantitative autoradiography and image processing; and Mr. E. S. Owens and Mr. R. Hyman for technical assistance with the experiments.

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the tumor site is assessed in relation to the exposure at systemic sites of potential toxicity. The latter presentation concerns local factors such as capillary density and intercapillary distances, diffusion through the extracellular space, cellular uptake, and drug biotransformation; the magnitude of drug exposure that can be achieved at or within a particlar tumor cell is assessed in relationship to the total plasma exposure.

MATERIALS AND METHODS

Animal Models and Experimental Procedure

Two experimental brain tumor models were studied: (*a*) metastatic Walker 256 carcinoma was induced by the intracarotid injection of tumor cells (9) (*b*) a chemically induced oligodendroglioma was obtained by the administration of ethylnitrosourea to pregnant rats (10,11). Tumor-bearing animals were studied when they began losing weight or demonstrated neurological signs of CNS involvement. The preparation of the animals for the studies of blood flow (F), blood to brain transport (K), and local glucose utilization (LCGU) were similar. The animals were anesthetized with pentobarbital, both femoral arteries and veins were catheterized, and blood pressure, rectal temperature, and blood gases were monitored. The studies of LCGU were performed in awake animals restrained by a plaster cast wrapped about their hind quarters as previously described (4).

Only a single ^{14}C-labeled compound was assayed per tissue and experiment with QAR. For the blood flow studies of F, ^{14}C-iodoantipyrine (IAP) was iv infused for 1 minute; for the investigations of blood to brain transfer, a plasma clearance constant, K, was determined. ^{14}C-alpha aminoisobutyric acid (AIB), a metabolically inert amino acid, was administered by iv bolus. For the local cerebral glucose utilization (LCGU) experiments, ^{14}C-2-deoxyglucose (2DG) was injected in a single iv bolus. In all cases, numerous (6 to 15) arterial blood samples were drawn during the experimental period and immediately placed in ice. As soon as possible, the cold blood samples were centrifuged and the plasma sampled and prepared for liquid scintillation spectroscopy.

The experiments were terminated by decapitation at either 1 minute (F), 10 minutes (K) or 45 minutes (LCGU) after initiating radioisotope injection or infusion. After decapitation the animal's brain was rapidly removed, frozen in liquid Freon II that had been chilled in liquid nitrogen to −40 °C, and stored at −80 °C until they were sectioned for autoradiography.

To prepare the tissues for QAR, 20-micron thick frozen sections of the brain were cut at −20 °C in a cryomicrotome (Minot custom microtome, Model CTI, International Equipment Corporation), placed on cover slips, dried rapidly on a warming plate at 65 °C, and mounted on pressed paper boards. After taking four slices for autoradiography, four to six slices 10 μ thick were cut for histological preparation. These tissue sections were stained with either hematoxylin and eosin or cresyl violet. Finally, a specific number of slices, usually 15 or 20, of 20-μ thickness were cut and discarded. Then the entire sectioning sequence was repeated until the frozen brain was completely sampled.

Sets of ^{14}C-methylmethacrylate standards (Radiochemical Centre, Amersham, England), which had been previously calibrated to reference 20-μ thick brain sections of known radioactivity, were placed in x-ray cassettes with the experimental tissue sections. Single-coated x-ray film (Kodak SB-5) was placed over the sections and standards in each cassette and exposed to their radiations for 7–14 days. To convert the x-ray film images to tissue radioactivity (nCi/g), the ^{14}C standards were read and a standard curve relating optical density to tissue radioactivity was generated for each film. Densitometric readings of the x-ray films were made with a computer-controlled scanning microdensitometer and stored. The radioactivity of the tissue was calculated for each densitometric measurement. These data were manipulated and expressed as a pseudocolor video image of the tissue section in which a range of values for F, K, and LCGU were coded to a color spectrum (6).

Blood Flow

The measurement of blood flow in these studies used iodoantipyrine (IAP) (3) and depends on the application of a working equation developed by Kety (16,17):

$$Ci(T) = \lambda k_o \int^{T} C_A(t) e^{-k(T-t)} dt \qquad \text{Eq 1}$$

where Ci(T) is the tissue concentration of radioactive IAP as determined by quantitative autoradiography at a given time, T, after introducing the tracer into the circulation, λ is the tissue–blood partition coefficient, C_A is the concentration of the tracer in arterial blood, and t is the variable time. Equation 1 is solved for k using the experimentally determined data for the other variables; k equals a constant that incorporated the rate of blood flow in the tissue as follows:

$$k = mF/\lambda \qquad \text{Eq 2}$$

In equation 2, F is the rate of blood flow per unit mass of tissue and m is a constant between 0 and 1 that represents the extent to which diffusional equilibrium between blood and tissue is achieved by the marker material during its passage from the arterial to the venous end of the capillary. Based on the work of Sakurada et al (3), m was taken to be equal to 1.0 and λ was taken to be equal to 0.8 for IAP. It has been shown that F is relatively insensitive to λ when $F < 0.6$ ml/g min for a "ramp" iv infusion of IAP over

a 60-second experimental period (unpublished observations).

Capillary Permeability

The movement or exchange of a particular solute across the capillary endothelium may be expressed in several ways (table 1). Measurements of solute activity or concentration in most in vivo experimental studies are limited to specific tissue, tissue region, or fluid such as blood, plasma, or CSF; measurements of extracellular and intracellular concentrations are usually not possible. The calculation of a unidirectional blood-to-brain transfer constant, K_1, from experimental data can be accomplished by the following equation, which was developed and used by Ohno et al (18):

$$K_1 = \frac{C_b(T)}{{}_0\int^T C_p(t)dt} \qquad \text{Eq 3}$$

where C_b is the concentration of the labeled material in the brain at the end of the experimental period, T, and C_p is the tracer's concentration in the plasma. If the plasma concentrations are kept constant, ${}_0\int^T C_p(t)dt$ equals $C_p \times T$.

To apply equation 3 for the calculation of K_1, one stringent experimental condition must be met; the unidirectional transport of tracer from brain to blood (backflux) must be negligible during the experimental period. This condition is satisfactorily met if the experimental period is comparatively short and extracellular fluid (ECF), levels remain low relative to C_p, or the test material is essentially trapped in the tissue by metabolic and/or transport processes. It is for the latter reason that we have employed labeled α-aminoisobutyric acid (AIB) rather than ^{14}C-sucrose (18) as an indicator of brain capillary permeability; this nonmetabolized amino acid is avidly concentrated by and trapped within brain cells by the small neutral amino acid transport mechanisms in the cell membrane (19,20).

The various expressions of capillary permeability listed in table 1 are interrelated. The transfer constant, K_1, is related to the extraction fraction (E) by

$$K_1 = E \cdot F \qquad \text{Eq 4}$$

and to the permeability-surface area product (PS) by the derivation of Crone (21) and equation 4

$$K_1 = F(1-e^{-PS/F}) \qquad \text{Eq 5}$$

If K_1/F is small (less than 0.2), equation 5 reduces to

$$K_1 \sim PS \qquad \text{Eq 6}$$

The PS product and K_1 value are expressed in terms of plasma clearance as volume per unit mass of tissue per unit time (cm³/gm min). For a two compartment system (ie, blood and tissue), the equilibration half time ($t_{½}$) is given by

$$t_{½} = V_e \frac{\ln 2}{K_1} \qquad \text{Eq 7}$$

providing plasma levels (C_p) are kept constant and V_e is the fraction of the tissue volume that exchanges or equilibrates with the blood.

Glucose Utilization

The measurement of rates of glucose utilization using the analog ^{14}C-deoxyglucose as the isotopic tracer is more formidable. The operational equation of the deoxyglucose method was developed by Sokoloff and used to calculate local cerebral glucose utilization (LCGU) (4). Sokoloff has shown for normal rat brain that terms including the individual rate constants from the model and appearing on the right side of both numerator and denominator in the operational equation become very small (5%–10%) if the experimental

TABLE 1.—Expressions of membrane permeability

Expression		Units	Comment
I	Permeability coefficient(P)	cm/sec	Physical characteristic of the membrane with respect to a particular solute
II	Permeability surface area product(PS)	cm³/g sec	Functional expression per unit tissue weight; surface area may not be known; units of clearance
III	Transfer constant(K)	ml/g min	Functional expression per unit tissue weight; surface area is not known; units of clearance
IV	Equilibration half-time($t_{½}$)	time	Functional expression dependent on the equilibration volume and efflux transfer constant
V	Extraction fraction(E)	none	Fractional loss from the blood during a single capillary passage

period is 45 minutes (5). The method also assumes that dephosphorylation of the labeled product, deoxyglucose phosphate, is negligible during this period.

If this analysis is correct and can be applied to pathological tissue such as brain tumors, the operational equation can be simplified to a form similar to equation 3 except for the plasma concentration of glucose (C_p) and the isotope correction factor. The latter term includes the ratio of the distribution space of deoxyglucose to glucose, a constant representing the steady-state fraction of glucose that, after phosphorylation, continues down the glycolytic pathways, and the Michaelis-Menten constant (K_m) and maximal velocity (V_m) of hexokinase for glucose and deoxyglucose respectively.

The isotope correction factor terms are written as a series of ratios that may be combined into a "lumped constant." The lumped constant has been determined for a variety of animals, anesthetized and unanesthetized, and has been found to range between 0.34 and 0.56 (5). It has not been determined for tumor tissue or pathological brain tissue, so the LCGU values obtained in the Walker 256 metastatic tumor or chemically induced neurogenic tumors are not quantitatively accurate. To circumvent this difficulty, the data are also expressed in terms of a relative glucose utilization rate, R′, by the following relationship:

$$R' = \frac{A_b(T)}{\int_0^T C_p^*/C_p(t)dt} \qquad \text{Eq 8}$$

where A_b is the ^{14}C activity measured in the brain at time T, and C_p^* and C_p are the arterial plasma concentrations of ^{14}C-deoxyglucose and glucose respectively. R′ incorporates the LCGU, the lumped constant, and the small corrections for the labeled precursor remaining in the tissue at time T, and for the lag in tissue equilibration with plasma. Equation 8 also assumes that dephosphorylation of the labeled product is negligible during the experimental period.

RESULTS

Blood Flow

A reduction in blood flow was observed in both tumor models (table 2, fig 1). Blood flow was severely reduced to < 0.1 ml/g min within the necrotic centers of several large metastatic tumors; blood flows ≥ 0.2 ml/g min were not associated with necrosis in the Walker 256 tumor. Blood flows 50% of normal were frequently observed in brain tissue surrounding large tumor masses. The chemically induced, well-differentiated oligodendrogliomas had blood flows similar to or slightly less than that measured for normal white matter. Necrotic centers were not observed within the well-differentiated tumors studied in this series despite their relatively large size.

TABLE 2.—Regional tissue blood flow; large and small tumors*

Description of region	Range of blood flows (ml/g min)†	
	Walker 256	Oligodendroglioma
Large tumors (2–5 mm diameter)		
"Necrotic" center	0.06–0.14	—
"Viable" portion	0.14–0.26	0.15–0.41
Peripheral edge	0.26–0.37	0.24–0.41
Small tumors (<1 mm diameter)		
In cortex	0.74–1.76	—
In pons	—	0.53–0.62
Brain tissue		
Adjacent to large tumors	0.37–0.74	0.33–1.16
Remote "normal" cortex	0.74–1.76	0.74–1.91
Remote "normal" white matter	0.26–0.44	0.22–0.41
Contralateral pons	—	0.62–0.71

*Preliminary observations; 2–3 animals per tumor.
†The lower limit of blood flow measurement was between 0.05 and 0.10 ml/g min.

Capillary Permeability

A variable increase in capillary permeability was observed in the Walker 256 tumors, whereas little or no increase was observed in the well-differentiated oligodendrogliomas (table 3, fig 2). Tumor size appeared to have a direct effect on capillary permeability of the Walker 256 metastasis; foci of tumor < 1 mm in diameter demonstrated little or no increase in permeability to AIB. Foci within large tumor masses were observed to have a 50- to 100-fold increase in the blood-to-brain transfer constant, k_1, for AIB. A consistent relationship between the k_1 values and the histological appearance of necrosis was not observed. The in-

TABLE 3.—Regional blood-to-brain transfer constants (K_1) for AIB: large and small tumors*

Description of region	Range of transfer constants (ml/g min)†	
	Walker 256	Oligodendroglioma
Large tumors (2–5 mm diameter)		
"Necrotic" center	0.020–0.042	—
"Viable" portion	0.024–0.10	< 0.003
Peripheral edge	0.010–0.026	< 0.003
Small tumors (1 mm diameter)		
In grey and white matter	< 0.003	< 0.003
Brain tissue		
Adjacent to large tumors	0.003–0.008	< 0.003
Remote "normal" brain	< 0.003	<0.003

*Preliminary observations; 2–3 animals per tumor
†The lower limit of measurement for the K_1 of AIB was 0.003 ml/g min.

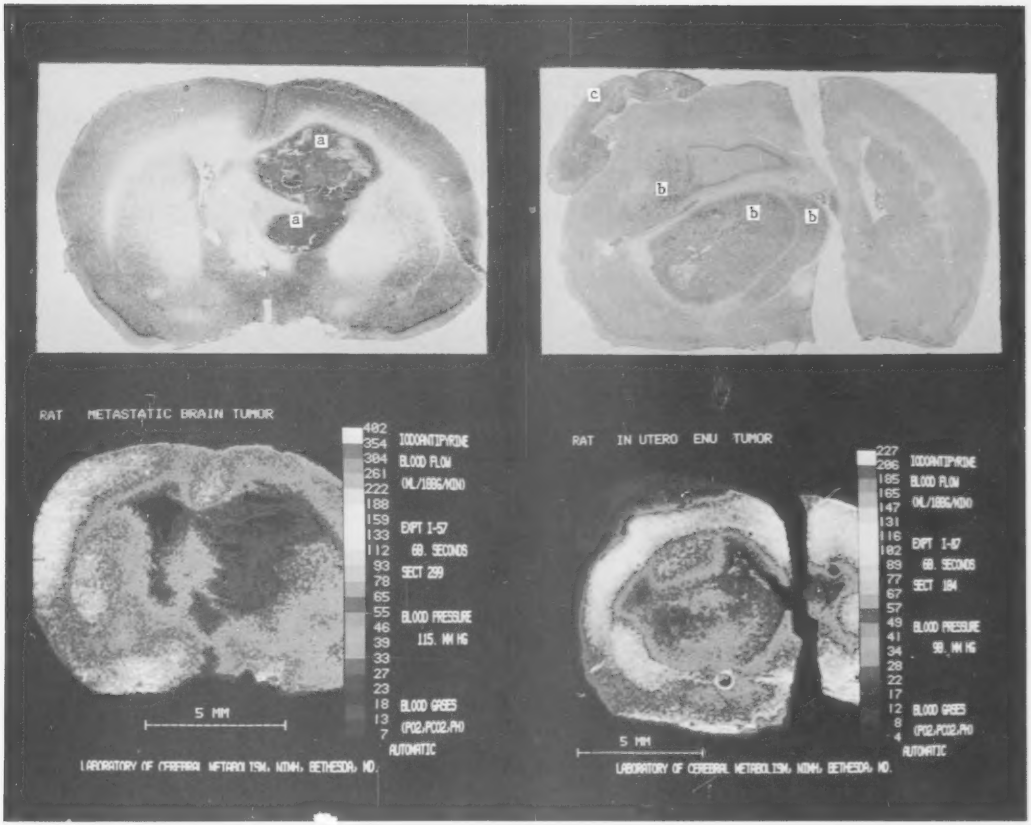

FIGURE 1.—Comparative histology and image analysis of blood flow determined by quantitative autoradiographic methods (1,24) in the Walker 256 metastatic tumor (a) and in the ENU induced oligodendroglioma (b) and schwannoma (c). Serial sections of brain, 20 microns thick, were cut in a cryomicrotome and processed for either quantitative autoradiography or histology; neighboring sections are shown above. The autoradiographic image produced on x-ray film was processed by a computerized microdensitometer; the optical density measurements (50 × 50-micron pixel) were converted to tissue radioactivity (nCi/g tissue wet weight) using calibrated ^{14}C-methylmethacrylate standards and subsequently expressed as blood flow (ml/100 g tissue wet weight/min). The blood flow values were coded to a color spectrum and a range of local blood flow values in the tissue was displayed as a pseudocolor image on a video monitor. Unfortunately, the gray scale of the black and white reproduction of the color spectrum is discontinuous between high and low blood flow values. Regional blood flow within tumor and surrounding brain tissue was determined by correlating a photograph of the histological section with a photograph of the video display; a range of blood flow values was obtained by matching the pseudocolor of the tissue image with that of the numerically coded spectrum at the right of the video monitor. The range of blood flow within different tissue regions is presented in table 2.

creased k_1 values within the areas of tissue necrosis probably represent a lower limit because tracer efflux or backflux from tissue to blood during the 10-minute course of the experiment may not be negligible; the transport mechanisms that effectively trap the tracer within brain cells would not be expected to function normally in grossly injured or dead tissue. A small increase in k_1 (two- to fourfold) was frequently observed within brain tissue immediately adjacent (0.5–1.0 mm) to the larger tumor masses (> 3 mm in diameter). The brain-tumor margins observed in the histological sections were usually sharply defined for both the Walker 256 metastasis and the well-differentiated oligodendroglioma.

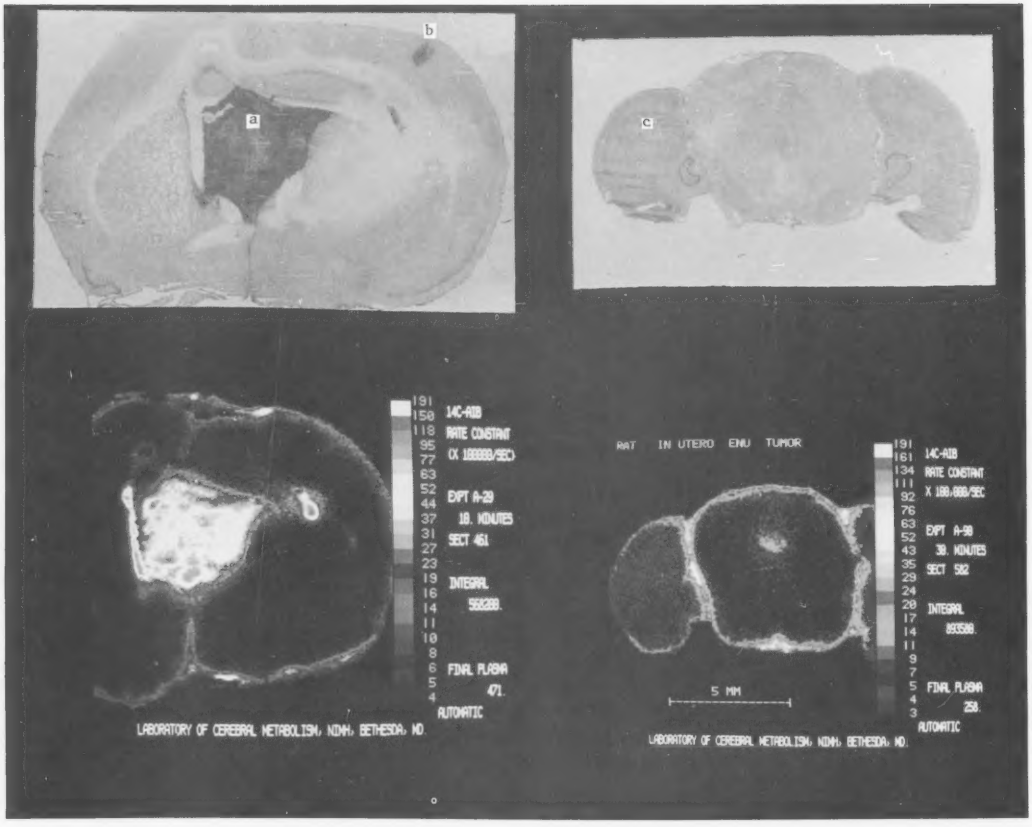

FIGURE 2.—Comparative histology and image analysis of capillary permeability (2,3) in the Walker 256 metastatic tumor (a,b) and in the ENU-induced oligodendroglioma (c). The experimental procedure was similar to that described in the legend to figure 1; capillary permeability is expressed in terms of a blood to brain transfer constant, K_1 (ml/g tissue wet weight/sec $\times 10^3$). The range of K_1 within different tissue regions is presented in table 3. Of particular note is the small focus of metastatic tumor (b), which demonstrates little or no increase in capillary permeability. The well-differentiated oligodendroglioma (c) demonstrates relatively "tight" capillaries in contrast to a similar size metastatic tumor (a).

Glucose Utilization

The limitations of quantifying glucose utilization within tumor tissue by the deoxyglucose method (4) were discussed in the Methods section. However, several significant observations can be made (table 4, fig 3). The two tumor models demonstrated clear differences. Relative glucose utilization, R′, was considerably higher within the histologically viable regions of the Walker 256 tumor than within the surrounding normal brain tissue, whereas R′ within the well-differentiated oligodendroglioma was uniform and similar to that of normal white matter. The autoradiographic pattern of the larger Walker 256 metastasis was grossly irregular, with sharply defined regions of very low and very high activity that closely followed the tumor outlines and demarcated the histologically nonviable from viable regions and tumor margins from surrounding brain tissue.

Several patterns of glucose utilization were observed within brain tissue surrounding the Walker 256 tumor. Very small microfoci of tumor could be detected due to the localization of very high R′ values; one focus was approximately seven cells in diameter. The increase in R′ was not limited to the tumor cells but involved a brain region 0.6 mm in diameter. This observation suggests that the microscopic foci of tumor can affect surrounding brain cells and result in their having

TABLE 4.—Deoxyglucose: relative glucose utilization (R') and local cerebral glucose utilization (LCGU) in large and isolated small tumors*

	Range of R' and LCGU (μM/g min)†			
	Walker 256		Oligodendroglioma	
Description of region	R'	LCGU	R'	LCGU
Large tumors	(2–5 mm diameter)		(2–5 mm diameter)	
"Necrotic" center	0.01–0.19	—	—	—
"Viable" portion	0.34–1.7	—	0.09–0.29	—
Peripheral edge	0.41–0.81	—	0.15–0.29	—
Small Tumors	(< 0.2 mm diameter)		(< 1 mm diameter)	
In grey and white matter	0.91–1.70	—	0.06–0.16	—
Brain tissue				
Cortex adjacent to large tumors	0.05–0.28	0.11–0.59	0.19–0.49	0.40–1.01
Cortex adjacent to small tumors	0.48–1.06	1.00–2.21	0.30–0.58	0.62–1.20
Contralateral "normal" cortex	0.34–0.67	0.70–1.39	0.30–0.65	0.62–1.34
Contralateral "normal" white matter	0.10–0.21	0.21–0.43	0.07–0.20	0.14–0.41

*Preliminary observations; 2–3 animals per tumor.
†The lower limits of measurement for R' and LCGU were 0.01 and 0.02 μM/g min.

increased glucose utilization. With larger metastatic foci (0.2–1.0 mm in diameter), this effect was variable or not observed. Very large tumor masses (2–5 mm in diameter) resulted in a decrease in R' and glucose utilization within surrounding brain tissue compared to contralateral brain regions remote from the tumor or in control animals.

DISCUSSION

The preliminary observations of regional blood flow, capillary permeability expressed as a blood-to-brain transfer constant, and deoxyglucose phosphate accumulation as an index or measure of relative glucose utilization in two brain tumor models have demonstrated considerable physiologic and biochemical heterogeneity. The ability to compare different tumor models as well as different animals with histologically similar tumors depends on the ability to obtain quantitative measurements that represent real values. This is particularly relevant to the deoxyglucose studies. The histologically viable-appearing Walker 256 cells had considerably higher R' values than the cells of the well-differentiated oligodendroglioma. The significance of this difference in R' cannot be directly correlated to differences in LCGU because the lumped constant for these tumors has not been determined (*see* Methods).

The variability that was observed in these studies does not appear to be limited to tumors with different classifications or origins. With the Walker 256 tumor model, considerable heterogeneity was observed between different metastatic foci as well as within a single focus. Furthermore, this diversity in the autoradiographic pattern within tumor tissue appeared to be partly related to tumor size. A similar variable pattern was observed in the localized autoradiographic measurements of blood flow and the blood-to-brain transfer constant of AIB. These observations suggest that it may be difficult to achieve uniform and adequate drug delivery to all regions of a brain tumor.

Relationship of Capillary Permeability and Blood Flow to Drug Delivery

The amount of drug, metabolite, or test substance that is brought to a particular mass of tissue is determined by the concentration of the free or unbound drug in the arterial blood or plasma (C_p) and the flow of blood (F) to the tissue mass. The amount of drug (A) that crosses the blood vessels or capillaries over time (t) is determined by the blood-to-tissue transfer constant, K_1, and C_p. Assuming linear or first-order transport kinetics, a simple relationship can be written:

$$\frac{dA}{dt} = K_1 C_p \qquad \text{Eq 10}$$

The integrated form of this relationship is similar to equation 3.

The relationships between K_1 and other expressions of permeability (table 1) were presented in equations 4–8. The transcapillary passage of drug or other substances within the brain or brain tumor depends on several parameters including the permeability coefficient of the blood vessels (P); the surface area of the

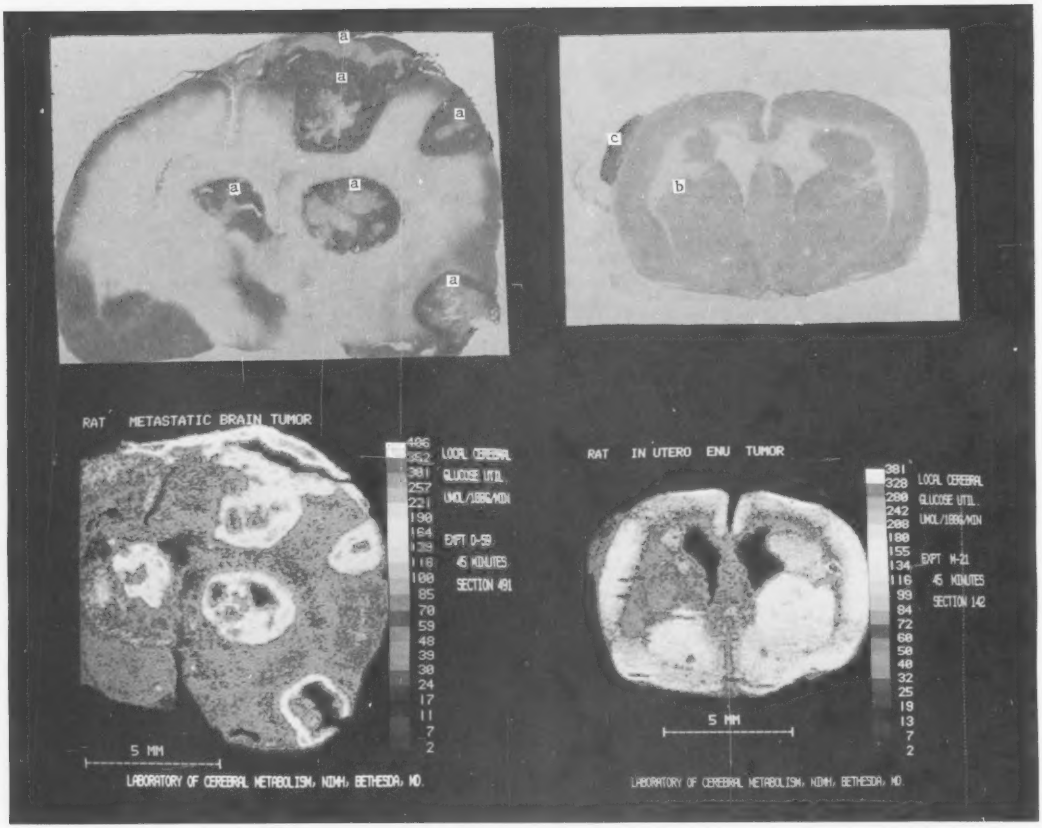

FIGURE 3.—Comparative histology and image analysis of glucose utilization (4,5,24) in the Walker 256 metastatic tumor (a) and in the ENU-induced oligodendroglioma (b) and schwannoma (c). The experimental procedure was similar to that described in the legend to figure 1. The glucose utilization values of the tumor tissue are not quantitatively accurate; *see* Methods. The range of relative glucose utilization, R', was calculated for the tumor tissue and is presented along with the range of LCGU of brain tissue in table 4.

blood vessels (S), which is partly a function of capillary density; the rate of blood flow through the vessels in a unit mass or volume of tissue (F); and the size of the tissue distribution space (V_e). The importance of F with respect to blood-to-tissue drug passage becomes apparent at low values of F, when K_1 approaches F. This situation was observed in the necrotic centers of the large metastatic Walker 256 tumors (tables 2 and 3). When the value of F approaches or is less than the PS product, blood-to-tissue transport is primarily dependent on the value of F and not on the PS product. This relationship is expressed by equation 5. However, the magnitude of PS and F depend, in part, on the same parameters; the magnitude of F is likely to depend on the capillary density or surface area (S) as well as on the velocity of flow through the capillary bed. If capillary diameter and length and the velocity of blood flow through different brain and/or tumor capillary networks are constant, S/F would also be constant in these regions. The relationships between K_1, PS, and F are discussed in greater detail elsewhere (24,25).

The above analysis was specifically limited to unidirectional transport across capillaries in order to emphasize the importance of very low blood flows with respect to drug and solute delivery to and clearance from the tumor mass. It does not consider the subsequent fate of drug or solute after it has crossed the blood vessels; this is discussed elsewhere (15).

Clinical Relevance of Capillary Permeability and/or Blood Flow to Chemotherapy

The relevance and usefulness of K_1, PS, $t_{½}$ and F to clinical problems associated with establishing effective chemotherapy protocols is demonstrated in table 5. A range of blood-to-brain transfer constants, K_1, or blood flow, F, is given in the left hand column; the corresponding time for reaching half-equilibration, $t_{½}$, within two exchangable tissue volumes (V_e) was calculated from equation 7 and is given in the two right-hand columns of the table. For comparison, the relative positions of several markers and drugs with respect to their K_1 values on a logarithmic scale in normal rat brain are also indicated.

The marker used in our quantitative autoradiographic studies was AIB. It was used to measure K_1 values greater than 0.003 ml/g min (table 3). If a particular drug were maintained at a constant level in the blood, and had a PS or K_1 value similar to that of AIB, ie, 0.001 ml/g min, and blood flow was not limiting ($K_1/F < 0.2$), the time required to achieve half-equilibration ($t_{½}$) with the extracellular fluid ($V_e = 0.15$) or the total tissue water ($V_e = 0.80$) would be 1.7 and 9.2 hours respectively. This calculation and the one below assumes that metabolism of the drug or test substance in the tissue was negligible during equilibration periods. Similarily, if a drug that permeates cell membranes rapidly (eg, CCNU) were maintained at a constant level in the blood, and the tumor region of interest had a blood flow of 0.001 ml/g min, the time required to achieve half-equilibration ($t_{½}$) with the extracellular fluid ($V_e = 0.15$) or the total tissue water ($V_e = 0.80$) would also be 1.7 and 9.2 hours. In recent studies, values of F as low as 0.002 ml/g min have been measured within the centers of flank tumor (unpublished observations). Thus the magnitude of F as well as K_1 can be an important determinant of overall drug and solute delivery to tumor tissue.

The highest K_1 values for AIB measured in the Walker 256 tumor were ~ 0.1 ml/g min (table 3); this value was similar to that obtained for blood flow measured within similar large tumor masses (table 2). In this case, blood flow (F) and/or capillary surface area (S) rather than the capillary permeability coefficient (P) could be the limiting parameter for drug and solute delivery to the tumor. In other tumor regions or tumor types, such as the chemically-induced well-differentiated oligodendroglioma, little or no increase in blood-to-brain or blood-to-tumor tissue transfer (K_1) for AIB was observed (table 3). In these cases, capillary permeability rather than blood flow may significantly impede drug delivery to the tumor, and the chemotherapist may wish to select drugs such as BCNU or CCNU that permeate the tumor blood vessels readily (table 5).

The expression of capillary permeability in terms of an equilibration $t_{½}$ is useful because it presents a time parameter that is clinically relevant and useful. The accurate calculation of $t_{½}$ requires a knowledge of V_e in

TABLE 5.—Blood-brain exchange and equilibration

K_1* or F† (ml/g min)	Markers‡	Drugs§	Equilibration half-time ($t_{½}$)¶ $V_e = 0.15$	$V_e = 0.80$
1	Water	BCNU CCNU	6.0 sec	33 sec
10^{-1}		Procarbazine	1.0 min	5.5 min
10^{-2}	Potassium	5-FU	10 min	55 min
10^{-3}	Urea, AIB, sodium, glycerol	Epipodophyllotoxin	1.7 hr	9.2 hr
10^{-4}	Mannitol		17 hr	3.9 day
10^{-5}	Sucrose, DTPA		7.2 day	39 day
10^{-6}	Albumin		72 day	385 day

*Blood-to-brain transfer constant.
†Blood flow
‡Literature values (22) and unpublished observations.
§Calculated from Levin et al (23)
¶$t_{½} = V_e \frac{\ln 2}{K_1}$ when the plasma concentration (C_p) is kept constant; V_e is the exchangeable volume—the fraction of the brain (tumor) volume that equilibrates with the blood (0.15 and 0.80 are equivalent to the extracellular space and total tissue water respectively). When K_1 approaches F in magnitude, F may be substituted for K_1 in the above equation.

addition to K_1 and F. However, this calculation does not consider drug metabolism or drug movement through the tissue and assumes a single tissue distribution compartment (V_e). Nevertheless, the concept of equilibration $t_{1/2}$ is simpler to grasp than that of P, PS, and K_1, particularly at the bedside. It must be emphasized that all these terms are related and it is possible to move from one to another as outlined in Methods.

CONCLUSIONS

The discussion by Vick et al (26) in response to the concept proposed by Levin et al (27) of an intact or partially intact blood-brain barrier within the zone of infiltrating tumor cells, the so-called "brain adjacent to tumor," raised a fundamental question concerning effective brain tumor chemotherapy: Has the limited clinical response to chemotherapy been related to inadequate delivery of drugs to the target cells due to capillary permeability (and/or blood flow) or to the inherent resistance of the tumor cells to our current chemotherapeutic agents? The answer may be provided by future investigations of drug distribution in an around CNS tumors using quantitative autoradiographic techniques and by studies of drug effectiveness against specific human brain tumor cell lines using tumor cloning techniques.

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Comparative Permeability of Different Glioma Models to Horseradish Peroxidase[1]

Dennis R. Groothuis,[2]* J. M. Fischer,[2] Nicholas A. Vick,[2] and Darell D. Bigner[3]

SUMMARY

Seven different experimental glioma models were studied to determine their capillary permeability to horseradish peroxidase. The models were the autochthonous ASV-viral model, intracerebral (ic) and (sc) injections of rat 9L and RG-2 tumor cell lines, the sc rat S-69C15 tumor cell line, and human glioblastoma cell lines in nude mice. The ASV-viral model showed an average HRP permeability of 63% of tumor volume, the ic RG-2 tumors were 100% permeable, and the ic 9L tumors were 41% permeable. The permeability of the marginal zone (brain-tumor interface) showed similar variation from group to group. In contrast, all of the sc tumors were 100% permeable regardless of the cell line used to create the tumors. Our results show the variability between these glioma models, and suggest that RG-2 ic gliomas and all sc gliomas should be optimal to assess the tumoricidal effect of drugs, because access to the tumor compartment from the vascular compartment is complete.

[Cancer Treat Rep 65 (Suppl 2):13–18, 1981]

The controversy about capillary permeability of primary intracerebral tumors — whether a vascular barrier exists, and if so, how extensive it is, and whether it is primarily within the body of the tumor or at the margin where brain and tumor cells intermingle — has yet to be resolved. On the one hand, Levin et al (1) have demonstrated that radiolabeled 5-FU reaches a concentration in mouse ependymoblastoma approximately 11 times that in normal brain. The concentration of 5-FU in the brain adjacent to tumor (BAT) was less than in the body of the tumor, but greater than in normal brain. In a study of BAT in intracerebral 9L and Walker 256 tumors, Levin et al (2) found that capillary exchange for ^{14}C-urea and radiolabeled sodium was 53% that of normal brain. They suggested that this reduction in permeability could be detrimental to the delivery of water soluble and rapidly binding drugs. On the other hand, electron microscopic studies of experimental (3–6) and human (7,8) brain tumors have revealed abnormally fenestrated endothelial cells that are permeable to biological tracers such as horseradish peroxidase (HRP) (5,9,10) and colloidal lanthanum (11). These studies suggest that, unlike normal brain, brain tumors lack an endothelial barrier to blood-borne substances, and that molecular size, lipid solubility, and the degree of ionization should not affect the permeability of brain tumor capillaries (12).

Because of this controversy, we systematically investigated the problem in seven different intracerebral (ic) and subcutaneous (sc) glioma models. We chose HRP as the biological tracer because it has a molecular weight of 44,000 and is much larger than drugs used in brain tumor chemotherapy. If HRP crosses tumor capillaries, then smaller compounds can be expected to behave similarly. HRP, which is readily visualized by light microscopy, is excluded from normal brain except in certain specialized structures (13). While we cannot provide truly quantitative data about the degree of HRP permeability into tumor, studies with light microscopy permit qualitative measurements and conclusions to be made that are impossible with the electron microscope because of the problem of specimen sample size.

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MATERIALS AND METHODS

Autochthonous tumors were induced by ic inoculation of neonatal Fischer-344 rats with 2 μl of avian sarcoma virus (ASV) (14). Ic rat tumors were produced by ic injection with RG-2 tumor cells (15) (8×10^4 cells in 2 μl) or 9L tumor cells (5×10^4 cells in 2 μl). Sc rat tumors were produced by sc inoculation of cultured cells as follows: RG-2 (5×10^4 cells in 2 μl), S-69C15, a glioma cell line derived from an ASV-induced tumor (15) (5×10^6 cells in 100 μl), and 9L (2×10^6 cells in 100 μl). A total of 75 tumor-bearing rats were used in these studies. Sc injections of human glioblastoma cell lines (H54, 8th passage; H79, 5th passage) were done in nude mice (16).

Ten control rats were used to verify that normal brain was impermeable to HRP. Some tumor-bearing rats in each group were not given HRP, which proved to be important because some tumors contained macrophages in areas of necrosis and randomly distributed tumor cells that contained endogenous peroxidase activity (17). Accordingly, only the extracellular HRP reaction product was considered when we estimated HRP permeability in the experimental animals (fig 1). In addition, some rats injected with virus or tumor cells did not have tumors at time of sacrifice. None of these rats had peroxidase activity outside the normally permeable specialized structures. Injection only transiently disrupted the normal blood-brain barrier to HRP (18).

Rats and nude mice were given a 30-second iv injection of 70 mg HRP (Sigma Chemical Co., Grade II) dissolved in 1 ml of normal saline. Between 1 minute and 24 hours after HRP injection, the animals were anesthetized with phenobarbital and sacrificed (tables 1 and 2). One group of ASV-inoculated tumor-bearing rodents received 70 mg HRP dissolved in 4.08 ml normal saline and administered continuously over 60 minutes into the tail vein with a Harvard pump. Rats with ic tumors were perfused through the aorta with heparinized saline and then with 3% glutaraldehyde in 0.1 M, pH 7.4 phosphate buffer. The brains were removed and kept in the fixative overnight.

After washing in 0.1 M phosphate buffer, they were cut coronally into 5 mm slabs and sectioned serially on a Vibratome™ at thicknesses of 50–70 μ. The sections were treated with diaminobenzidine by the method of Malmgren and Olsson (19). Some sections were counterstained with Nissl stain or hematoxylin and eosin, while others were mounted without counterstain. We tried intravascular perfusion of sc tumors, but fixation was consistently superior by immersion in 3% glutaraldehyde. Except for the method of fixation, the sc tumors were handled identically to the ic tumors.

The degree of HRP permeability was estimated semiquantitatively by planimetric estimates of HRP-positive tumor to HRP-negative tumor; the results are expressed as percentage of tumor permeable to HRP. The marginal zone (brain-tumor interface) was scored as positive if HRP was found entirely around the perimeter of the tumor in the brain adjacent to tumor.

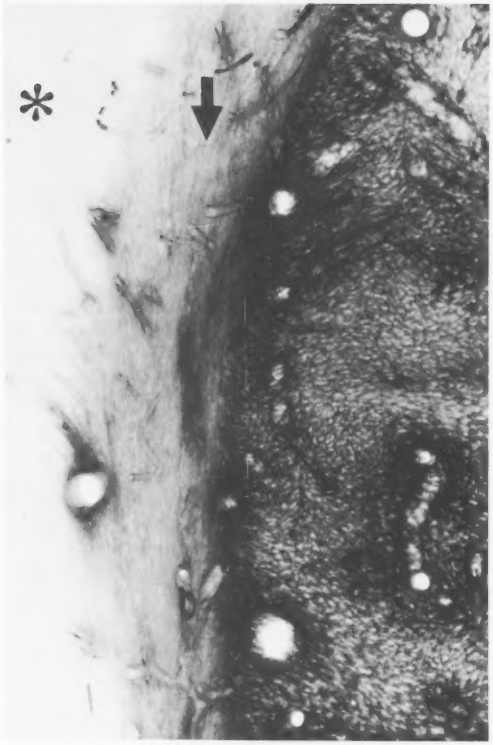


FIGURE 1.—Photomicrograph of the edge of an RG-2 glioma. HRP appears black. The tumor (on the right) contains extracellular HRP in a dense, lacy network. The marginal zone (arrow) is also permeated with HRP, while more distant brain (*) is free of HRP. No counterstain was used. Magnification = 40 ×.

RESULTS

ASV-Virus Intracerebral Gliomas

Forty-six tumors were studied (table 1). Variability in HRP permeability was found both within an individual tumor and between tumors. At 5 minutes postinfusion, some tumors in each group were 100% permeated by the HRP; most tumors also had marginal zones that contained HRP. However, in each

TABLE 1.—Permeability of intracerebral tumors

Tumor model	No.	Survival time	Permeability		
			Mean	Range (percent)	Marginal zone
(HRP administered by iv bolus injuction)					
ASV	9	1 min	11	(0–60)	0
	2	2 min	35	(0–70)	0
	2	5 min	50	(0–100)	50
	5	10 min	50	(20–100)	40
	7	20 min	50	(5–100)	57
	15	30 min	63	(0–100)	66
	3	60 min	53	(40–80)	100
	3	24 hr	13	(0–40)	33
RG-2	4	15 min	100	—	100
	3	30 min	100	—	100
	7	60 min	100	—	100
9L	3	15 min	10	(0–20)	0
	13	30 min	41	(5–80)	0
(HRP administered by continuous iv infusion)					
ASV	11	60 min	73	(5–100)	90

TABLE 2.—Permeability of subcutaneous tumors

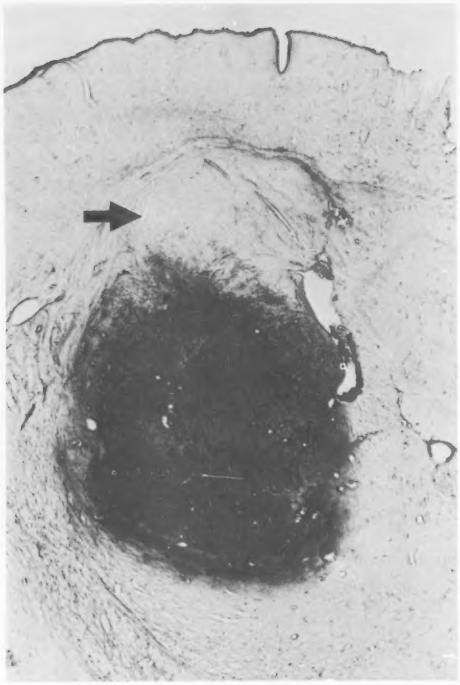
Tumor cell line	No.	Survival time	Permeability (percent)
RG-2	15	10–60 min	100
9L	16	15–30 min	100
S69-C1-5	7	1–60 min	100
Human GBM	5	5 min	100

of these groups tumors ranged in their degree of permeability from 0%–100%, and the pattern of HRP penetration into the tumor and the marginal zone was very irregular and unpredictable. In some instances the center of a tumor did not contain HRP even though the periphery and marginal zone were completely permeated. There was no apparent correlation between the histological type of the tumor (anaplastic, pilocytic, gemistocytic, or polymorphic) or the location of the tumor (subependymal, cortical, or cerebellar) and HRP penetration. However, there seemed to be a relation between tumor vascularity and HRP penetration (figs 2 and 3).

The ASV-inoculated rats given HRP by continuous infusion showed greater HRP permeability in both tumor and the marginal zone, even though the total amount of HRP administered was equivalent to the amount administered as a bolus. However, even continuous infusion of HRP failed to make all tumors 100% permeable. One of these rats contained three distinct tumors. One of two anaplastic gliomas and a pilocytic glioma were 100% permeated, both within the tumors and in their marginal zones. The other anaplastic glioma, which was relatively avascular, contained HRP in only 5% of its tumor mass.

Cell-Injected Intracerebral Gliomas

These tumors were more homogeneous from model to model (table 1). The RG-2 gliomas were all 100% permeable between 15 and 60 minutes; the marginal zones of these tumors were completely permeated (fig 1). In contrast, 9L tumors, which averaged 41% permeable, showed an even lower degree of HRP penetration than the ASV-viral tumors, and in no instance was the BAT completely permeated. Of note is the fact that the marginal zones of the ASV-virus and RG-2 tumors were similar; the transition from tumor to tumor-free brain often occurred within a single high power microscopic field. In the 9L tumors, the BAT as defined by Levin (2) extended for some distance from the tumor, and the degree of perivascular extension of tumor cells was striking and different from the other ic tumor models. In some instances there were collections of perivascular cuffs of tumor cells in the hemisphere opposite the tumor mass.



FIGURES 2 & 3.—Adjacent sections from an ASV-induced glioma. The section in figure 2 was reacted for HRP but not counterstained. The section in figure 3 was counterstained with Nissl. The lower part of the glioma contains dense HRP-reaction product, while the upper avascular portion (arrows) is free of HRP. This tumor illustrates the regional variability to HRP permeability seen in most ASV-induced gliomas. Magnification = 5 ×.

Subcutaneous Gliomas

Regardless of the cell line of origin, all sc tumors showed 100% permeability (fig 4, table 2). Even though the RG-2 tumors tended to become centrally necrotic when small, unlike the 9L tumors or the sc human tumors in nude mice, permeability was complete.

DISCUSSION

There were three unexpected results from this study. The first was the variable permeability to HRP of the ASV-virally induced gliomas. This is important because this model produces a histological spectrum of tumors (14) that is thought to correspond closely to human malignant gliomas (20). Previous studies of HRP permeability in this model demonstrated the presence of the reaction product between HRP and diaminobenzidine at the electron microscopic level (9,10). In retrospect, sampling of the tumor and marginal zone was inadequate for generalization about the entire tumor. Vick et al (12) have concluded that ASV-induced gliomas in dogs that were permeable to HRP were invariably anaplastic. This conclusion was not corroborated by the present study in rats, which failed to demonstrate a consistent relationship between cellular morphology and HRP permeability. The only morphological feature that seemed to correlate with HRP permeability was vascularity — the number of tumor vessels per unit area (figs 2 and 3). If this model does indeed closely mimic the behavior of human tumors, they can be expected to show similar variability in permeability. There may be some human malignant gliomas that are 100% permeable to large, hydrophilic compounds, and in these tumors permeability may not be a factor in chemotherapy. The problem then remains to identify which human tumors are completely permeable, and to distinguish them from those that are incompletely permeable.

The second unexpected observation was the variability between tumor models (table 1). In the RG-2 model, all tumors were completely permeable both

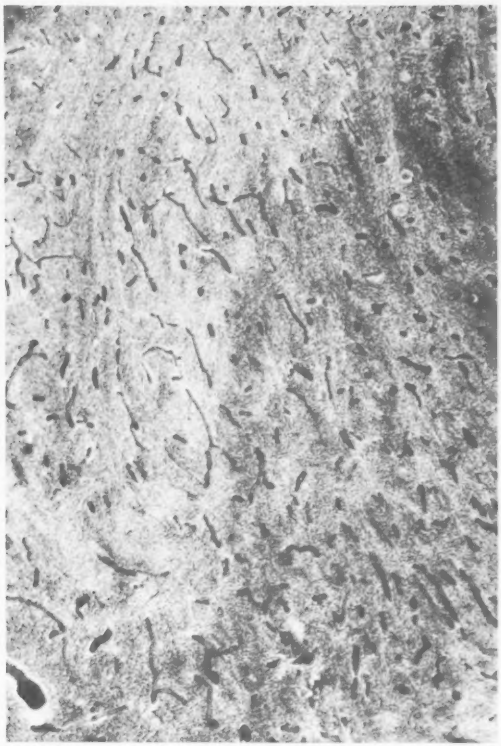


FIGURE 4.—Subcutaneous 9L tumor. The section was reacted for HRP but not counterstained. The HRP, which appears black, was found throughout the tumor. The tumor was not perfused, so blood vessels containing red calls are prominently stained as well. Magnification = 4X.

within the body of the tumor and at the marginal zone (fig 1). In the 9L model, although there was tumor-to-tumor variation, the bodies of the tumors were not well-permeated by HRP and in no instance was the interface of brain and tumor (BAT) permeated by HRP. In the ASV-virally induced tumors, the variation among individual tumors was most striking. Some tumors were completely permeable while others were completely impermeable to HRP. It seems clear also that the terms marginal zone and BAT, as they have evolved in different laboratories, cannot be used synonymously. We agree with the estimate of Levin et al that the BAT contains 10%–15% tumor cells (2). Our histological observations support their isotope studies that showed the BAT in intracerebral 9L tumors has a reduced permeability, at least compared to the tumor proper. Conclusions about the permeability of one tumor model cannot be generalized to all tumors.

The third and perhaps most important result of this study is the observation of complete HRP permeability in sc tumors. The difference between sc and ic tumors is brought into sharp focus by the two 9L models. Ic they averaged 41% HRP permeability, while sc all were 100% permeable. This observation may be of importance if one wishes to test the effect of a chemotherapeutic agent in vitro against glioma cells, because in sc-grown gliomas a permeability barrier does not appear to exist. It is tempting to speculate that the sc tumors recruit blood vessels from adjacent sc tissues that are normally permeable to HRP. They have abundant pinocytic vesicles and lack tight junctions between endothelial cells. In contrast, ic tumors may recruit brain capillaries that normally lack fenestrae, have few pinocytotic vesicles, and have tight junctions between endothelial cells. The greatest puzzle is why some brain tumor capillaries have fenestrae; one must assume they are either transformed due to soluble factors derived from the neuroectodermal tumor cells or are themselves neoplastic (21).

Collectively, our results suggest that much remains to be learned about the factors that influence capillary permeability in brain tumors and that the blood-brain barrier in ic gliomas must still be considered a factor in the delivery of chemotherapeutic drugs. Clearly, it is not entirely absent, as we so firmly believed on the basis of our previous ultrastructural studies (12). How much of a factor it actually is remains to be seen, and only truly quantitative studies can provide the information needed.

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Drug Delivery to CNS Tumors[1]

Victor A. Levin,[2,*] Herbert D. Landahl,[3] and Clifford S. Patlak[4]

The fundamental tenet governing pharmacology is that the active drug moiety must get to the appropriate site of action in "adequate" amounts to exert its biological effect. For cancer chemotherapeutic agents, this tenet must be modified to include the condition that the active drug moiety must get to the appropriate site of action at a specific time in the life cycle of the cancer cell. Further, in order for the drug to exert its cytotoxic effect, the cancer cell must not repair the damage produced by the drug.

As discussed at this conference (1), drugs that normally cross the blood brain barrier (BBB) exert somewhat more antitumor activity than drugs that are restricted in their BBB transport. The purpose of this presentation is twofold: to explain why drugs that cross the BBB are more effective against CNS tumors and to emphasize factors that influence drug delivery to all solid tumors.

CNS and extra-CNS tumors are quite similar. Blood vessels feeding the tumor and capillaries within the tumor vary considerably in shape and size; shunting of blood from one region to another is common and produces relative hypoperfusion in some regions and hyperperfusion in others. There is tremendous variation in intercapillary distances (ICD) within tumors. When these distances are great, diffusion of oxygen and metabolic substrates are compromised, which leads to cellular necrosis. Figure 1 is a schema of a typical tumor capillary that shows the decrease in mitotic activity (eg, growth fraction) with increasing distance from the capillary. Ultimately, necrosis occurs when the capillary to cell distance exceeds 150 μm (eg, ICD > 300 μm) (2).

There is an important difference between CNS tumors and extra-CNS tumors. In CNS tumors, neoplastic cells are capable of extensive infiltration into normal brain tissue that precedes extension of the typical tumor vasculature. As a result, cells that have high proliferative potential are theoretically inaccessible to drugs that do not normally cross the BBB, which is part of the reason these drugs are less effective than drugs that cross the BBB.

CAPILLARY TO CELL TRANSPORT

Physical factors that influence drug delivery from capillary to cells at varying distances from capillaries are summarized in table 1. The contribution of each of these factors to the steady-state tumor drug exposure integral (C_T or $C \times T$) was the subject of a previous publication (3), from which figures 2 and 3 were adapted. They show the influence of high capillary permeability (1×10^{-4} cm/sec) to low permeability (1×10^{-6} cm/sec), the influence of high and low cellular/extracellular partitioning (f = 1 and 6), intracellular drug half-life (ICF $t_{1/2} = 5$ and 50 min), and tumor blood flow (0–1 ml/g/min). It was assumed in the modeling that blood velocity remained constant in different regions of the tumor. Therefore, low blood flow was associated with low capillary surface area and a large ICD; conversely, high blood flow was associated with high capillary surface area and a small ICD. Extracellular fluid (ECF) drug half-life was varied between 50 and 400 minutes and had no significant influence on C_T.

Two models were considered. The Krogh model assumes that tumor capillaries can be described as rows of parallel cylinders, and the well-mixed model assumes that there is homogeniety of capillaries around cells such that all cells are the same distance from a capillary. The two models represent the upper and lower extremes of the model; the actual in vivo

TABLE 1.—Factors influencing drug delivery to solid tumors

1. Blood flow
2. Capillary surface area and permeability
3. Cellular permeability
4. Cellular/extracellular drug ratio
5. Extracellular drug half life
6. Intracellular drug half life
7. Drug diffusion coefficient

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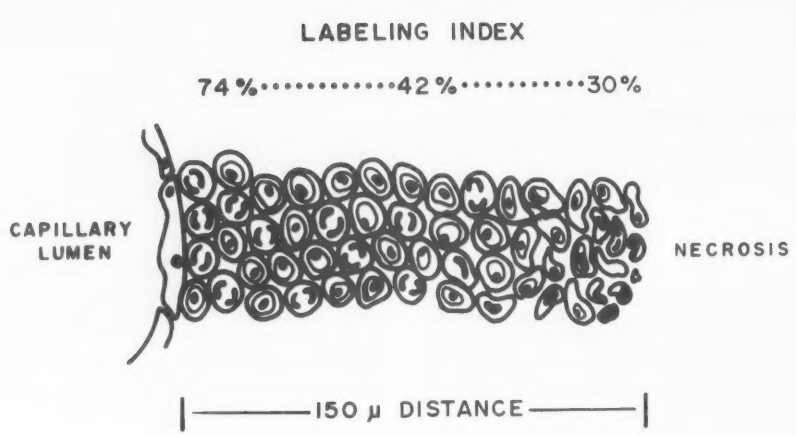


FIGURE 1.—Relationship of a capillary to surrounding tumor cells, cellular viability, and mitotic activity labeling index (2) as a function of distance from the capillary lumen.

situation must lie somewhere between the two extremes. At low capillary permeability, the two models produce almost identical C_Ts and their differences, even at high capillary permeability, are modest.

Blasberg's paper presented data obtained by an elegant radioautographic method and showed the enormous variation in both tumor blood flow and permeability (4). Table 2 summaries some of the data we obtained for ICD, plasma volume, blood flow, and capillary permeability for the ic and sc 9L gliosarcoma (VA Levin, DL Wright, and J Csetjey, unpublished observations, 1979). This table shows the tremendous heterogeneity in this tumor type, with 40-fold differences in ICD, 18-fold differences in plasma volume, 700-fold variation in blood flow, and 100- to 200-fold variation in capillary permeability. Undoubtedly, there is as much or greater variation in these parameters among different types of tumors as there are within this relatively uniform syngenic tumor. Such variation surely occurs in human tumors. Mantyla recently measured blood flow in human tumors using the xenon washout technique and found a greater than tenfold variation in blood flow between lymphomatous tumors and adenocarcinoma and squamous cell tumors (5).

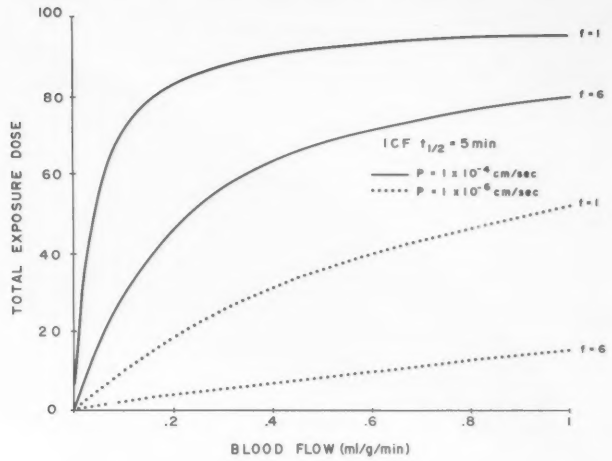
DRUG DELIVERY FROM WELL PERFUSED AND POORLY PERFUSED MACROSCOPIC REGIONS

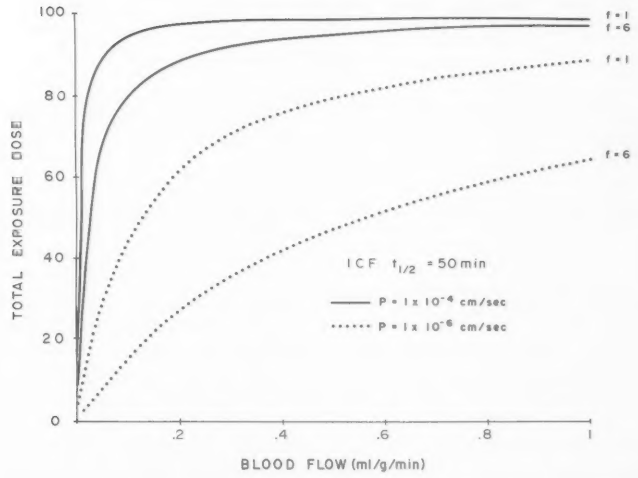
It is clear that drug delivery from capillary to cell will be severely compromised in regions with low blood flow, low capillary permeability, and/or for drugs that have short ICF $t_{1/2}$ and/or high cellular/ECF partition ratios. Thus many drugs such as the antitumor antibiotics and methotrexate that do not cross the BBB will have poor penetration and little or no antitumor activity. In addition, low levels of drug penetration might hasten the emergence (overgrowth) of drug-resistant clones or stimulate phenotypic modification (ie, gene amplification).

One method of overcoming this problem would be to infuse drugs intravenously for long periods of time or to use drugs with long plasma half-lives to achieve higher drug levels in hypoperfused tumor regions by diffusion of drug from well-perfused regions. As with capillary to cell transport, the rate-limiting factors are the same as listed in table 1. In addition, because the process of diffusion is slow and the distances drugs must diffuse may be on the order of millimeters to cen-

TABLE 2.—Variation in plasma volume, blood flow, and permeability in subcutaneous 9L tumors less than 2 cm diameter

	ICD	Plasma volume	Blood flow	PS_{urea}	PS_{miso}	PS_{bleo}
	(μm)	(ml/g)	(ml/g/min)		($\times 10^{-4}$ min^{-1})	
Median	85	0.049	0.02	18	30	58
Range	5-205	0.004-0.071	0.001-0.72	1.17-288	12.6-168	1.6-160
No.	370	20	62	28	19	20





FIGURES 2 and 3.—Total drug exposure dose as a function of blood flow, peremability coefficient (P), cellular/extra cellular drug concentration (f), and intracellular drug half-life (ICF $t_{1/2}$). The tumor was assumed to have an average capillary radius of 9.5 μm, an extracellular fluid volume (V_e) of 0.27 ml/g tumor, and an ECF $t_{1/2}$ ~ 400 minutes. Blood flow is related to intercapillary distance and can be approximated so that 1 ml/g/min is approximately 16 μm, 0.4 ml/g/min is approximately 40 μm, and 0.02 ml/g/min is approximately 126 μm.

timeters, potential benefits to this approach also will be limited (3). Figure 4 shows the depth of diffusion achieved as a function of the overall drug half-life (composite of extracellular and cellular half-times) for 50% of the capillary drug concentration (3,6). The two diffusion coefficients chosen cover a representative range for the anticancer drugs that are normally used to treat CNS tumors.

It is apparent from figure 4 that this approach has its limitations, particularly in view of the fact that the long drug infusion may produce significant systemic toxicity unless some method of late rescue can be

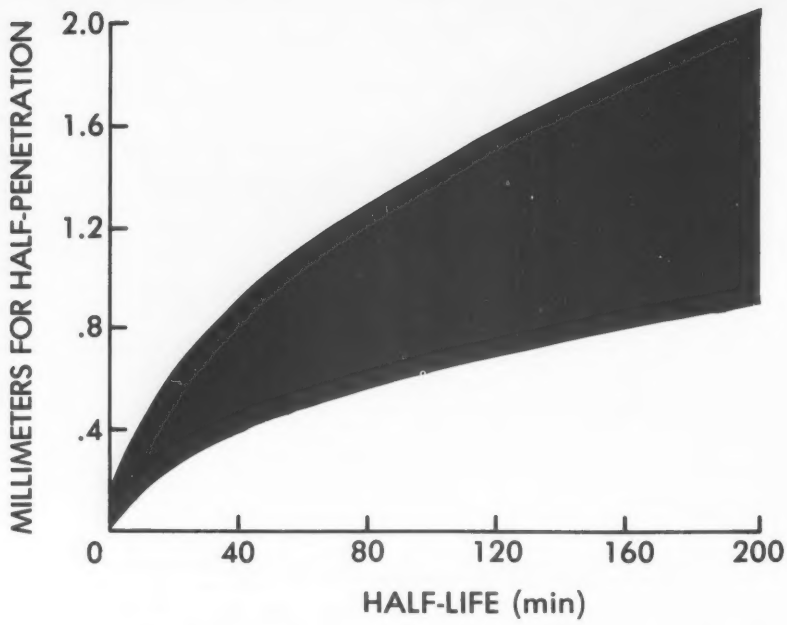


FIGURE 4.—Depth of half penetration (50% of "outside" or permeable region exposure dose) at equilibrium as a function of drug half-life. The model presumes diffusion perpendicular to a plane.

invoked to preferentially prevent systemic toxicity without neutralizing tumor cytotoxicity.

THE USE OF HEURISTIC MODELING TO PREDICT ANTITUMOR ACTIVITY IN VIVO[5]

The nitrosoureas BCNU and PCNU chemically transform by two general pathways to produce the diazoalkylahydroxide considered to be the active species that causes DNA-DNA and DNA-protein cross-linking and nitrosourea antitumor activity (8,9), or to produce carbamoylating agents that do not seem to correlate as closely with antitumor activity. Weinkam and Deen assumed that antitumor activity among the nitrosoureas would correlate with the amount of alkylating species produced. Because the reactive intermediate reacts in milliseconds after formation to alkylate the nearest nuceophile, the overall chemical transformation rate in aqueous solution at pH 7.4 should reflect quite closely the rate of formation of this intermediate. In addition, if the rate of this chemical transformation is considerably slower than the rate at which this drug enters from the extracellular to the cellular environment, it can be assumed that the rate of formation in the cytosol parallels the rate of chemical transformation in the extracellular environment. Thus the rate of nitrosourea product formation intracellularly is closely related to its rate of transformation in a phosphate buffer at pH 7.4 and 37°.

When these assumptions were used to compute the amount of cytotoxic nitrosourea product formed and were related to the in vitro log cell kill of 9L cells, it was found that both drugs produce precisely the same log cell kill as a function of μmoles of product formed (fig 5). As measured by CFE assay, in vivo BCNU and PCNU produce exactly the same log cell kill based on μmol of drug/kg animal weight (fig 6).

Modeling the steady state C_T for BCNU and PCNU for 9L tumors as a function of blood flow and parameters that we have been previously determined in vivo (4) shows that for equimolar drug doses PCNU has a significantly lower C_T than BCNU (fig 7). If C_T were in fact the determinant of antitumor activity for these two nitrosoureas, based on figure 6 one would have expected that the two C_T curves would have been the same. Because we know that the BCNU $t_{1/2}$ in

[5]We are indebted to Robert Weinkam and Dennis Deen for allowing us to use their concepts of nitrosourea activity and experimental in vivo data (R Weinkam and D Deen, manuscript in preparation), and to Mark Rosenblum for making available in vivo to in vitro colony-forming efficiency assay data for BCNU and PCNU (M. Rosenblum. Unpublished observations).

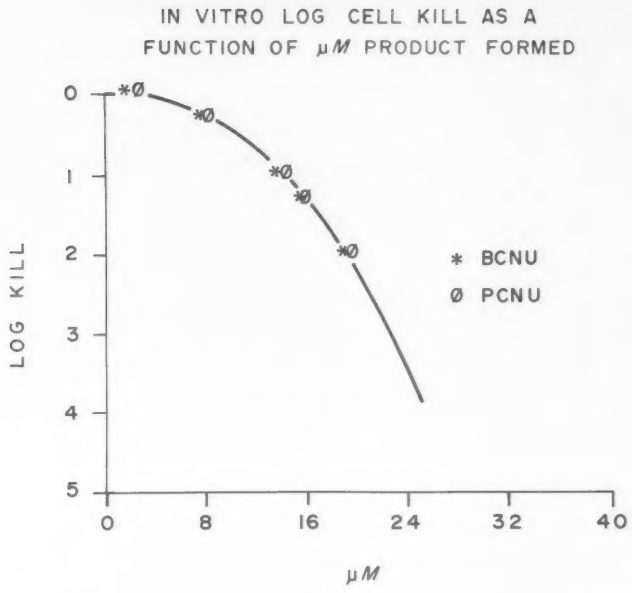


FIGURE 5.—Alkylating product formed was computed from the time-dose and half-life values for BCNU and PCNU.

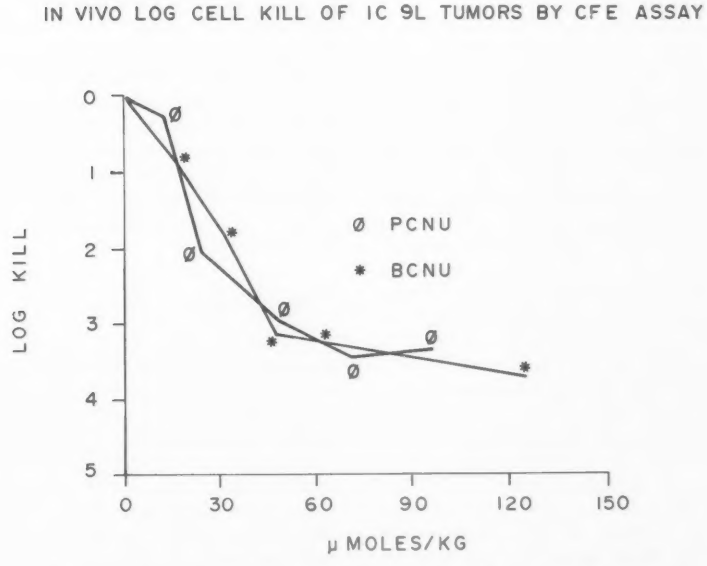


FIGURE 6.—Intracerebral 9L tumor cell kill determined by CFE assay for different doses of BCNU and PCNU.

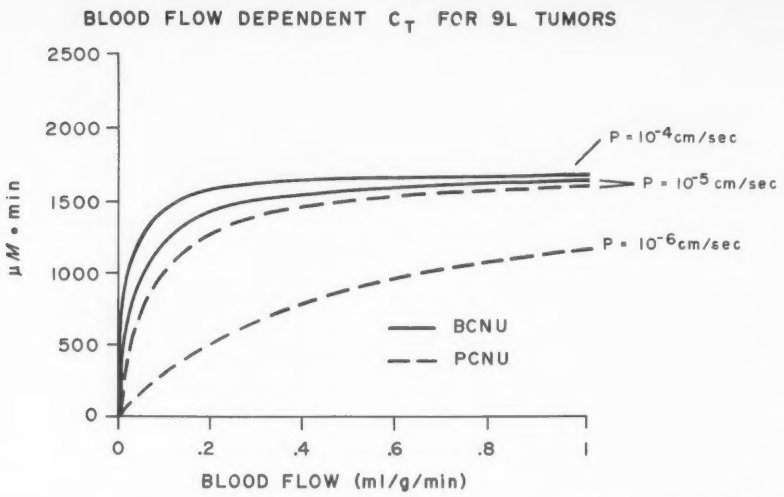


FIGURE 7.—Total drug exposure as a function of blood flow and permeability for BCNU and PCNU in 9L tumors in rats. BCNU and PCNU pharmacokinetics in rats and ECF and ICF half-lives were used to compute these curves. The highest anticipated capillary permeability coefficient (3) and a coefficient tenfold less were used because some tumor regions will have reduced permeability.

aqueous buffer is 49 minutes and the $t_{1/2}$ for PCNU is 26 minutes, we can extend the steady-state C_T model to compute steady-state product formed as a function of capillary permeability and blood flow.

Figure 8 shows product formation as a function of blood flow and capillary permeability. The permeability values chosen represent a value for the highest permeability attainable and a value 1/10 of the maximum, the latter value representing tumor regions that may be poorly perfused because of low blood flow and low capillary density. This suggests that at high blood flow PCNU would be a superior drug and would be expected to produce significantly more cell kill than BCNU. However, at lower permeability the amount of product formed and the cell kill obtained would be less. Against the 9L tumor in situ there may be sufficient heterogeneity in capillary permeability and blood flow such that the average product formed from PCNU would be similar to the product formed from BCNU, which may explain why at similar doses both drugs produced the same log cell kill in vivo.

In addition to helping the development of drug analogs and increasing our understanding of the physical factors that govern drug delivery, this modeling suggests advantages of one drug over another that might have clinical relevance. In this instance it is apparent that PCNU would clearly be the drug of choice if a tumor had relatively high blood flow and relatively uniform and elevated permeability. While supportive data are not extensive, the studies suggest that PCNU would be a superior nitrosourea for Hodgkin's disease (6) and other tumors with high blood flow (> 1 mg/g/min). On the other hand, for a tumor with relatively low blood flow, BCNU would be less affected by variation in capillary permeability and would be superior for these tumors.

Pharmacokinetic data suggest that a combination of BCNU and PCNU might be superior to either drug alone for brain tumors; PCNU would achieve a much higher log cell kill in the areas very near the capillary and BCNU would be more effective against cells further from the capillary and in regions with low permeability.

CONCLUSION

In this discussion we have demonstrated the tremendous heterogeneity in blood flow and permeability within a single tumor type and the value of computer simulated models that predict drug delivery to cells at varying distances from capillaries and to macroscopic tumor regions at varying distances from well-perfused tumor regions. This type of heuristic modeling can be of significant value, as demonstrated by the analysis of the BCNU and PCNU pharmacokinetics applied to in vitro and in vivo log cell kill.

Clearly, pharmacokinetic considerations alone will not lead to a cure for brain tumors or any other cancer.

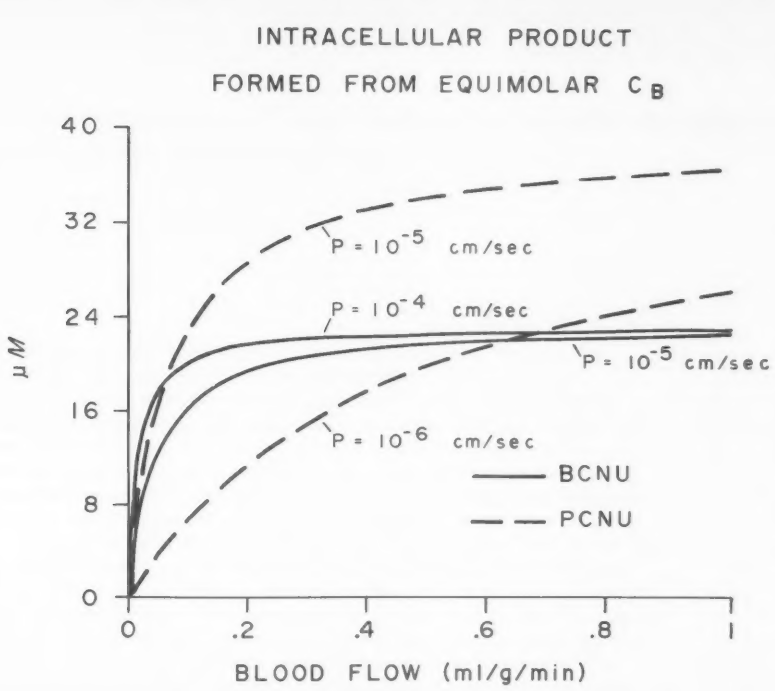


FIGURE 8.—Total ICF alkylating product(s) formed in 9L tumors in rats for BCNU and PCNU. The same parameters used in figure 7 were used here, except that the ICF and ECF $t_{1/2}$ were used to compute from the exposure integral, C_T, the product formed.

Other factors of tumor heterogeneity such as clonal sensitivity to anticancer agents remain the major problem. Specificity and selectivity of anticancer therapy should be the goals of all pharmacologists and therapists, but realizing those goals will clearly require consideration of the physical characteristics that govern drug delivery to the active site within a cell.

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Intra-arterial Infusions of Drugs and Hyperosmotic Solutions as Ways of Enhancing CNS Chemotherapy

J. Fenstermacher[1,*] and J. Gazendam[1,2,3]

SUMMARY

The use of intra-arterial (ia) infusions of drugs to enhance their target tissue uptake and minimize their systemic delivery and toxicity was examined by mathematical analysis and computer modeling. In addition, the effects of intra-carotid infusions of hyperosmotic solutions on blood-brain barrier permeability and the transfer of solutes between blood and CNS were evaluated with rats. In virtually all situations, at least some increased target site drug delivery and decreased systemic drug distribution will be obtained by an ia infusion. Significant ia infusion advantages for the target tissue will be realized in situations where either the rate of blood flow through the infused artery and the associated capillary bed within the target site is very low or the rate of systemic drug transformation or excretion is very high. If the drug's rate of transformation-excretion within the target tissue is appreciable, factors that produce a long residence or transit time for these molecules within the target site, namely low blood flow, high capillary permeability, and large distribution space, will decrease systemic drug delivery with an ia infusion. The study of blood-brain barrier (BBB) opening by ia hyperosmotic infusion indicated that BBB permeability is increased 30 to 50-fold in various brain areas over short periods of time (≤ 6 minutes) and returns to normal over the next hour or so with this technique in the rat. Clear regional and subregional variations in opening were also observed.

[Cancer Treat Rep 65 (Suppl 2):27-37, 1981]

For a number of years, arterial infusions have been considered a good way of delivering drugs to specific tissue sites. This approach has intrigued chemotherapists as a clinical strategy to maximize drug delivery to the diseased tissue and minimize drug distribution to normal tissue. Because of the blood-brain barrier's (BBB) general restriction to drug entry into the central nervous system (CNS), infusions of chemotherapeutic agents into the major arteries that perfuse the CNS seem to be particularly attractive for the treatment of brain and spinal cord lesions. Yet, despite the appeal of this suggestion, very little work has been done to rigorously test this proposition for either the brain or other organs.

One of the purposes of the present paper is to explore by mathematical analysis the fundamental principles of circulation, blood-tissue exchange, metabolism, and excretion that govern the advantages of intro-arterial (ia) infusions over other methods of drug administration and to illustrate the interaction of these various factors by computer modeling. The mathematical analysis is based on the work of Eckman et al (1); the computer model findings are taken from other studies from this laboratory (2,3).

Rapoport and co-workers (4–6) have suggested that enhanced drug uptake by brain tissue could be obtained by initially "opening" the BBB with an arterial infusion of a hyperosmotic solution and subse-

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quent intravascular administration of the drug during the period when the BBB is "opened". Experimental data on the magnitude and time-course of hyperosmotic BBB opening, which were determined by quantitative autoradiography in rats with the CNS capillary permeability marker, α-aminoisobutyric acid, are presented as the second part of this study.

DEFINITIONS

Several terms are used repeatedly throughout the text, and are defined as follows.

1. *Target tissue* or *site*—the tissue or site within the diseased organ to which the drug must be delivered for effective chemotherapy;
2. *Target* or *infused artery*—the single artery that serves the target site whether drug administration is intra-arterial or intravenous (see the assumptions for further elaboration on this definition);
3. *Systemic artery* or *arteries*—all arteries other than the target one;
4. *Systemic organs, tissues,* or *sites*—all organs, tissues, and sites of the body except the target tissue or site;
5. *Arterial* or intra-*arterial (ia) infusion*—an infusion into the target artery only;
6. *Venous or intravenous (iv) infusion*—an infusion into any vein;
7. *Drug integral or exposure* (X)—the concentration-time product (C × T) of the drug in the blood or tissue;
8. *Drug transformation-excretion*—the combination of all processes that irreversibly remove the material from the circulation (metabolism, chemical breakdown, tissue binding, and excretion) into one general function;
9. *Drug distribution advantage* (R_d)—ratio of drug exposure obtained for a particular tissue site after beginning an ia infusion (X_{IA}) to that obtained after beginning an iv infusion (X_{IV}) or, expressed mathematically,

$$R_d = X_{IA}/X_{IV} \qquad \text{Eq 1}$$

In connection with equation 1, the following relationships always hold: $R_d \geq 1$ for the target site and artery and $R_d \leq 1$ for the systemic arteries and organs. For the target site, the larger the value of R_d the greater is the drug delivery to the target tissue and the ia infusion advantage; for systemic sites, the smaller the value of R_d the lesser is the systemic drug delivery and the greater is the ia infusion advantage. Finally, at least some increased drug delivery to the target site and decreased systemic drug distribution will be obtained by an ia infusion in virtually all cases, but the magnitude of this gain may be very small.

MATHEMATICAL ANALYSIS OF IA INFUSIONS AND R_d

The following evaluation of ia infusions as a means of optimizing drug delivery to target and systemic tissues is based on the work of Eckman et al (1). In their mathematical treatment the following assumptions were made.

1. The kinetics of drug distribution within the body can be effectively represented by linear compartmental analysis.
2. The rate coefficients that describe the various transport processes and transformation-excretion in the body are constant (first-order) and remain so over the entire range of drug concentrations and throughout the period of drug exposure.
3. The actions of the drug within all the tissues are directly proportional to the concentrations and concentration-time integrals.
4. In all cases, the same number of drug molecules (N_i) are administered.
5. The target or infused artery perfuses only the target tissue and the target tissue or site receives no blood from other arteries; this is the best case for an ia infusion since blood from other arteries will dilute the drug before reaching the target tissue's capillaries and lower the advantage.

With their distribution model, Eckman et al (1) have mathematically established that the drug exposure at any site within an organ is directly proportional to the concentration-time integral in the arterial blood perfusing that site, either X_{IA} or X_{IV}. The distribution parameters that function within the extravascular part of the tissue, including capillary permeability, are independent of the route of drug administration and interact with the drug molecules presented to them by the arterial system in the same way, regardless of the infusion site; this part of the system can not tell an ia-infused drug molecule from an iv-infused one and vice versa. This means that R_d is completely determined by X_{IA} and X_{IV}.

The ia and iv exposures for the target and systemic arteries depend on the probability that the infused drug molecules will pass through them some time during the course of distribution. To illustrate this, consider separate ia and iv infusions of N_i drug molecules. For an ia infusion, N_i molecules will pass through the target artery; if fewer do, R_d will be less than the ideal case. For an iv infusion, N_i or fewer molecules will pass through the target artery at least once during the existence of the drug in the body, the exact number depending on the drug's rate of systemic transformation-excretion. If the rate of systemic transformation-excretion is high, only a few of the iv-infused drug molecules will ever reach the target artery, X_{IV} will be relatively small and R_d for

the target artery will be large. If the rate of systemic transformation-excretion is very low, virtually all of the iv-infused drug will eventually reach the target artery, X_{IV} and X_{IA} will be nearly equal, and R_d for the target artery will approach 1 (no advantage).

For systemic tissues, the advantage of an arterial infusion comes from the reduction in the magnitude of X_{IA} and not changes in X_{IV}. With an iv infusion, N_i molecules will arrive in the right heart and be distributed throughout the body; however, depending on the fate of the drug molecules during their first passage through the target artery and tissue, N_i or fewer will be delivered to the right heart following ia administration. Thus the arterial infusion advantage that is established for the systemic organs is produced by the target tissue's rate of drug transformation-excretion. This is just the opposite advantage dependency as the target tissue has.

Target Site R_d

As shown previously, (1-3), X_{IA} and X_{IV} are independent of the drug's infusion rate in a linear system. The validity of this independence for biological systems and pharmacological studies is a prerequisite for the acceptance of the following discussion on the quantitation of R_d.

With the above proviso, the best drug administration schedule for demonstrating the magnitude of R_d, namely, a rapid bolus injection, can be chosen, and the results generalized to all cases. Figure 1 presents hypothetical blood concentration curves for the target and systemic arteries with both ia and iv bolus injections. Each set of curves can be readily broken into a first passage integral (X_a and X_v for ia and iv injections respectively) and a recirculation integral (X_{ra} and X_{rv} for ia and iv injections respectively), and R_d can be expressed as:

$$R_d = (X_a + X_{ra})/(X_r + X_{rv}) \qquad \text{Eq 2}$$

The Stewart-Hamilton principle, which applies during the first passage of a substance through the arterial circulation, states that blood flow through a particular part of the arterial system during this time is equal to the total amount of the administered material (A) divided by the first passage concentration-time integral within that vessel. Using this principle, Eckman et al (1) generated the following expression for determining R_d from equation 2:

$$R_d = \frac{1 + \alpha\,(CO)\,\phi_a}{\alpha\,(1-p_l) + \alpha\,(CO)\,\phi_v} \qquad \text{Eq 3}$$

where CO is the cardiac output; F_a is the rate of blood flow through the target artery; α is F_a/CO or the fraction of CO that flows through the infused artery; ϕ_a is X_{ra}/A; ϕ_v is X_{rv}/A; and p_l is the probability that the drug will be transformed-excreted in one passage through the lungs ($0 \le p_l \le 1$).

The advantage of increased drug delivery to the target organ, therefore, is determined by the values of CO and F_a, two parameters that are known or measurable for most species, and the two recirculation integrals. The two latter parameters can be experimentally measured easily from bolus injection experiments with the drug. If F_a is low, α will be small, and the denominator in equation 3 approaches zero, yielding an R_d for the target site much greater than 1 (a significant ia infusion advantage). If transformation and/or excretion are slow processes, the recirculation integrals will be big and ϕ_a and ϕ_v will be large and similar in magnitude, thereby dominating equation 3 and causing R_d to approach 1 (no ia infusion advantage). Finally, if pulmonary clearance is large (p_l approaches 1), the terms ϕ_v, ϕ_a, and $(1-p_l)$ become very small, and R_d for the target organ is much greater than 1.

Eckman et al (1) also developed an equation for determining R_d that does not require knowledge of X_{ra} and X_{rv} but of the rates of transformation-excretion in the lungs and systemic tissues. This expression is:

$$R_d = \frac{1 + (1-\alpha)(p_s/\alpha)}{a-p_l} \qquad \text{Eq 4}$$

where p_s is the probability that a molecule of drug will be lost from the body during a single passage through the entire systemic circulation, including the lungs but excluding the target artery. The terms in equation 4 which make the numerator large (a small α or a large p_s) or the denominator small lead to a large target artery R_d. In summary, an arterial infusion advantage for the target organ will be realized in a situation where the rate of blood flow through the target artery is very low and/or the rate of systemic drug transformation-excretion is very high.

Systemic Tissue R_d

In order to deliver less drug to the systemic circulation and organs during and after an ia infusion, there must be an irreversible loss of the agent from the circulation during its initial passage through the target tissue. This means, first, that a significant number of drug molecules must move across the capillaries of the target tissue and, second, that a reasonable portion of these molecules must subsequently be transformed and/or excreted within the target tissue and not return to the circulation as parent compound. Thus, as mathematically expressed by Eckman et al (1) for systemic tissues,

$$R_d = 1-p_b \qquad \text{Eq 5}$$

REPRESENTATION OF ARTERIAL CONCENTRATION CURVES FOLLOWING BOLUS INTRA-ARTERIAL AND INTRAVENOUS INJECTIONS OF A HYPOTHETICAL DRUG

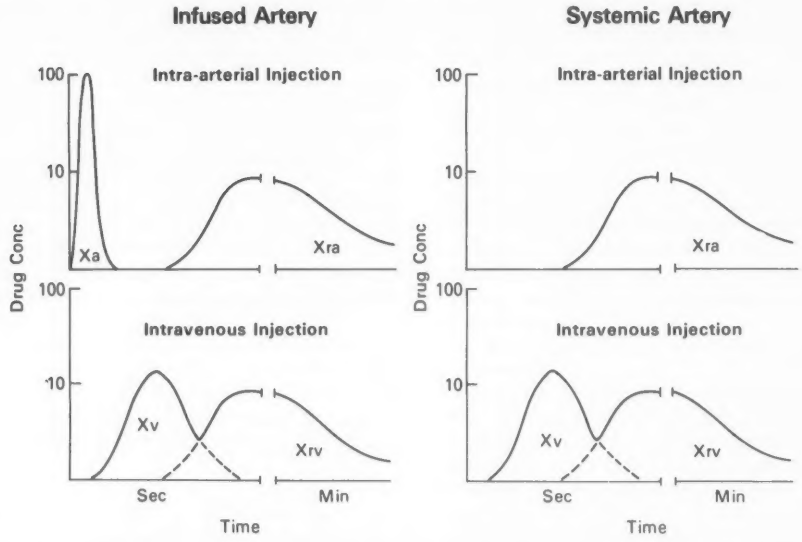

FIGURE 1.—Representations of infused (target) and systemic arterial concentration curves following hypothetical bolus ia and iv injections of a model drug.

where p_b is the probability that a drug molecule will be lost permanently from the circulation during a single passage through the infused artery and target site. For systemic tissues, the smaller the value of R_d is, the greater the advantage becomes.

The probability of irreversible drug loss within the tissue depends on the target tissue's rate constant for transformation-excretion (k_b, which is expressed as a fraction per unit time) and the amount of the compound that actually enters the target tissue during the first passage of the injected bolus and remains there at any time thereafter. The latter parameter can be shown to be the same as the mean transit time (1), $\bar{t}$, which equals the tissue distribution volume (also known as the tissue: blood partition coefficient, λ) divided by the blood flow. Therefore, the following equations hold:

$$p_b = k_b\bar{t} \qquad \text{Eq 6}$$

and

$$R_d = 1-k_b\bar{t} \qquad \text{Eq 7}$$

Equation 7 shows that factors that produce a longer residence or transit time for the drug molecules at the target site—large tissue distribution volume and low blood flow—decrease the systemic delivery of the chemotherapeutic agent following ia administration, providing that $K_b$is not effectively zero within the target tissue.

In contrast to the factors that provide the advantage of increased drug delivery to the target site, those that set the ia infusion advantage for the systemic tissues are the target tissue's distribution and disposition parameters.

COMPUTER MODELING OF IA INFUSIONS AND R_d

The computer model used for the predictions of ia and iv drug distribution has been previously described (2,3). In brief, the model consists of a number of blood and tissue compartments linked in a physiologically appropriate arrangement. Each compartment was scaled for blood flow and size. The major assumptions are virtually identical to those given above.

The variables in the model are the site of infusion (the target artery or a central vein), the rate of infusion, the individual tissue:blood partition coefficients (λ) or

distribution ratios of the drug, the rate of tissue perfusion or blood flow, the blood-to-tissue exchange or extraction fraction (E, a measure of the drug's capillary permeability), and the rate of drug transformation-excretion. Two parameters—the amount of hypothetical drug (100 mg) and the degree of intermixing of blood from the target artery with other arterial blood before reaching the target site (10% mixing)—were kept constant.

Arbitrary Conditions

Initially, an arbitrary set of conditions was chosen, and the distribution time courses of the model compound for both ia and iv infusions were generated. Then one parameter of the model was altered and the subsequent effects of this change on the ia and iv distributions were determined. Because a computer model such as this produces a tremendous amount of data, only a small portion of the total output can be given. The data presented are the drug exposures of the target artery (the left carotid), the target organ (the portion of the brain that is perfused by the left carotid artery), the systemic artery (a general term used hereafter to refer to all arterial compartments other than the target artery), and the combined bone marrow, splanchnic, and lymphoid tissues (representative systemic tissues with a high potential for toxicity and subsequently referred to in the text as the marrow only).

The arbitrary conditions were: (*a*) a drug infusion of 25 mg/min for 4 minutes; (*b*) a tissue:blood λ for all organs of 1.00, (*c*) an E of 0.8 for all tissues; and (*d*) a transformation-excretion half-time ($t_{½}$) of 30 minutes for all tissue compartments. Figure 2 presents the arterial and tissue time courses of drug concentration for both ia and iv infusions under the arbitrary conditions.

As previously mentioned, there will almost always be some drug delivery advantage with an ia infusion, provided the infused artery primarily perfuses the target site and not normal tissue. This advantage is shown in the four panels of figure 2 by the differences between the solid (IA) and dashed (IV) curves and by the associated X values. The X_{IA}:X_{IV} ratios for both the target artery and target tissue are 2.66 (= R_d); for the systemic artery and the marrow, this ratio equals 0.96. In the arbitrary case, the absolute values of R_d are not so important in themselves since they only reflect the chosen values of the parameters. The key thing, rather, is how changes in a particular variable alter R_d relative to the arbitrary value.

The data in figure 2 also indicate that only the target and systemic artery exposures need to be considered in the discussion of drug delivery advantages since the target and systemic tissue exposures are always directly proportional to their respective arterial values. For example, when X_{IA} for the target artery is 354, the X_{IA} for the target tissue is 335 and the tissue:artery ratio is about 0.95 (= 335/354); when X_{IV} for the target artery is 133, the X_{IV} for the target tissue is 126 and the tissue:artery ratio is again 0.95 (= 126/133).

Infusion Rate

The effects of the intravascular infusion rate upon tissue and blood exposures were determined by holding the amount of drug infused constant and varying the rate and duration of infusion. Table 1 presents these data and shows that the infusion rate does not affect the exposures as long as the total amount of infused drug is the same. This point was previously cited in the mathematical treatment and used there to justify the case which was chosen to best illustrate the ia infusion advantage, the bolus injection.

Target Organ R_d

Equations 2, 3, and 4 were presented to show the parameters that affect the arterial infusion advantage for the target site ($R_d \gg 1$). They indicate that the advantage is initially established during the first passage of the injected agent through the target artery and depends in magnitude on the rate of blood flow through the target artery and cardiac output, but subsequently it is diminished by the recirculation of the drug throughout the body. If the drug has large recirculation integrals (X_{ra} and X_{rv}), namely, if the drug is not rapidly transformed and/or excreted, the first pass exposure advantage becomes trivial relative to X_{ra} and X_{rv}, and R_d approaches 1. Based on this analysis, the effects of changes in F_a and the rate constant of whole body transformation-excretion (k_w) on R_d were assessed by the computer model.

Two examples of the alterations in the arterial exposures caused by varying F_a from the arbitrary conditions (F_a = 0.52 ml/g-min), which would be expected to affect X_a and X_v, are given in table 2. As foretold by equations 3 and 4, a low F_a increased the target artery's exposure for an ia administration and gave a relatively high R_d (= 4.3), whereas a high F_a decreases X_{IA} of the target artery and the related R_d (= 1.8).

In contrast to F_a, the rate of transformation-excretion affects the recirculation portion of the total integral. Table 3 presents model data that were generalized by changing k_w (arbitrary value: $T_{½}$ = 30 min, k_w = 0.0233 min^{-1}). When k_w was increased sixfold, all Xs were smaller, but for the target artery R_d was clearly increased since X_{IV} (= 269) was more sharply diminished than X_{IA} (=52). When k_w was reduced to 25% of the arbitrary value, all Xs were increased and R_d approached 1. The range of effects of k_w on the recircula-

TIME COURSE OF BLOOD AND TISSUE CONCENTRATIONS OF MODEL DRUG FOR EITHER AN INTRA-ARTERIAL OR INTRAVENOUS INFUSION OF 25 mg/min FOR 4 MINUTES

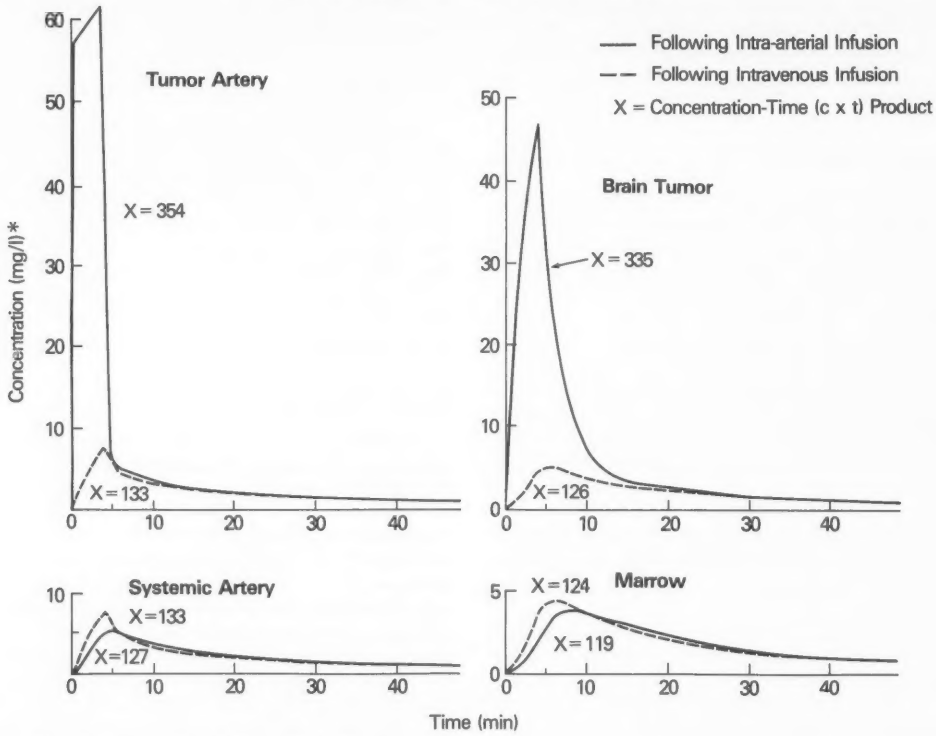

FIGURE 2.—Computer-generated time courses of model drug concentrations in the target and systemic arteries and the target and marrow tissues for ia and iv infusions of 25 mg/min for 4 min

TABLE 1.—Effects of infusion rate on blood and tissue exposures after either an ia or iv infusion of a model drug

Infusion schedule (mg/min)	Mode of infusion or mode ratio	Exposure (mg-min/liter) or ratio			
		Target artery	Target tissue	Systemic artery	Marrow
100 × 1 min	ia	354	335	127	119
	iv	133	126	133	124
	ia/iv	2.66	2.66	0.96	0.96
25 × 4 min*	ia	354	335	127	119
	iv	133	126	133	124
	ia/iv	2.66	2.66	0.96	0.96
1 × 100 min	ia	354	335	127	119
	iv	133	126	133	124
	ia/iv	2.66	2.66	0.96	0.96

*Arbitrary case as presented in the text and figure 2.

TABLE 2.—The effects of target artery blood flow (F_a) on arterial exposures after either an ia or iv infusion of a model drug

F_a (ml/g-min)	Mode of infusion or mode ratio	Exposure (mg-min/liter) or ratio		
		Target artery	Systemic artery	Target/systemic
0.26	ia	576	123	4.7
	iv	133	133	1.0
	ia/iv	4.3	0.92	—
1.04	ia	243	130	1.9
	iv	133	133	1.0
	ia/iv	1.8	0.98	—

TABLE 3.—The effects of the rate of transformation-excretion on arterial exposures after either an ia or iv infusion of a model drug

Rate constant (min^{-1})	Mode of infusion or mode ratio	Exposure (mg-min/liter) or ratio		
		Target artery	Systemic artery	Target/systemic
0.14	ia	269	42	6.4
	iv	52	52	1.0
	ia/iv	5.1	0.80	—
0.006	ia	581	355	1.6
	iv	358	358	1.0
	ia/iv	1.6	0.99	—

tion integrals and the exposures are obvious from these data.

The amount of drug that is transformed-excreted is also affected by altering the concentrations of the molecules in the tissues, because the rate of this process is the product of k_w and the various tissue drug concentrations. The concentration of a chemotherapeutic agent in a particular tissue depends upon not only the arterial integral but also the values of λ and E of the drug in the tissue. Table 4 shows the effects of changing all the tissue λs upon arterial exposures. For the target artery, R_d becomes greater as the tissue λs increase as a result of a marked rise in the amount of drug transformed and excreted and a sharp fall in X_{IV}.

Varying E for all tissues simultaneously also produced changes in the arterial exposures (table 5), the highest value of R_d (2.8) was obtained with the largest value of E. Because the possible range for E (0–1.0) is limited whereas that for λ is much broader, larger changes in the tissue's rate of drug transformation-excretion and R_d can be achieved with the model by varying λ than E.

Systemic Tissue R_d

Equation 7 indicates that lower systemic drug delivery will occur with an ia infusion if the $k_b\bar{t}$ product is appreciable, that is, if significant irreversible loss of the compound from the circulation occurs within the target tissue. The systemic artery ia/iv ratios in tables 2–5 indicate that noteworthy advantages in reduced systemic drug delivery ($R_d \leq 0.9$) occur with high rates of overall metabolism (table 3) and large tissue:blood partition coefficients (table 4) and, possibly ($R_d \cong 0.9$), with low target artery blood flow (table 2). In equation 7, $\bar{t}$ is a function of λ and F_a, whereas the rate constant, k_b, reflects transformation-excretion.

To further demonstrate the extent that R_d for the systemic tissues is set by the target tissue's distribution and disposition parameters, two of these variables for just the target site, λ and E, were altered in the model; the results are presented in tables 6 and 7 respectively. The systemic artery exposures show a definite dependence on the target tissue's λ; at high values of λ, a clear systemic tissue drug delivery advantage is evident. On the other hand, with the parameters used for this hypothetical drug and case, variation of the target site's E up to the maximum (E = 1) produced only a small difference in the systemic artery R_d.

The distribution data generated by the computer model corroborate the equations and conclusions presented in the mathematical section about the advantages of ia infusion, namely, enhanced target organ and/or reduced systemic tissue drug delivery. To reiterate, a better delivery of drug to the target site will be achieved with an ia infusion than with a comparable

TABLE 4.—The effects of the tissue-blood partition coefficients (λ) of a model drug on the arterial exposures after either an ia or iv infusion

Value of λ for all tissues	Mode of infusion or mode ratio	Exposure (mg-min/liter) or ratio		
		Target artery	Systemic artery	Target/systemic
0.25	ia	583	356	1.6
	iv	360	360	1.0
	ia/iv	1.6	0.99	—
4.0	ia	281	54	5.2
	iv	64	64	1.0
	ia/iv	4.4	0.84	—

TABLE 5.—The effects of the blood-tissue extraction fraction (E) of a model drug on the arterial exposures after either an ia or iv infusion

Value of E for all tissues	Mode of infusion or mode ratio	Exposure (mg-min/liter) or ratio		
		Target artery	Systemic artery	Target/systemic
0.1	ia	584	357	1.6
	iv	368	368	1.0
	ia/iv	1.6	0.97	—
1.0	ia	344	117	2.9
	iv	122	122	1.0
	ia/iv	2.8	0.96	—

TABLE 6.—The effects of the target tissue-blood partition coefficient (λ) of a model drug on the arterial exposures after either an ia or iv infusion

Value of target tissue λ	Mode of infusion or mode ratio	Exposure (mg-min/liter) or ratio		
		Target artery	Systemic artery	Target/systemic
0.25	ia	362	136	2.7
	iv	137	137	1.0
	ia/iv	2.6	0.99	—
4.0	ia	329	102	3.2
	iv	120	120	1.0
	ia/iv	2.7	0.85	—

TABLE 7.—The effect of the blood-target tissue extraction fraction (E) of a model drug on the arterial exposures after either an ia or iv infusion

Value of target tissue E	Mode of infusion or mode ratio	Exposure (mg-min/liter) or ratio		
		Target artery	Systemic artery	Target/systemic
0.1	ia	357	131	2.7
	iv	135	135	1.0
	ia/iv	2.6	0.97	—
1.0	ia	354	127	2.8
	iv	133	133	1.0
	ia/iv	2.7	0.96	—

iv infusion if a low rate of blood flow through the infused artery or if a high rate of systemic tissue transformation-excretion exists. Moreover, lower systemic drug delivery will be found with an ia infusion if an appreciable amount of the agent enters the target tissue during its first passage through that organ and subsequently is lost from further circulation by transformation-excretion within the target site.

Chen and Gross (8) reviewed the pharmacokinetics of five cytotoxic drugs—adriamycin, bleomycin, 5-FU, melphalan, and MTX—and analyzed the advantages of administering them intra-arterially for a number of different organ systems, following the approach of Eckman et al (1). They also examined the clinical data from ia infusion studies with these five drugs. On theoretical and pharmacokinetic grounds, 5-FU, melphalan, and MTX would be expected to distribute advantageously when given ia; however only 5-FU has been shown clinically to yield an improved response rate in the treatment of head and neck, progressive liver, and colorectal cancers when given ia. Blasberg (9) has suggested that BCNU, thio-TEPA, and, possibly, procarbazine, are good candidates for brain tumor chemotherapy by intracarotid infusion because of their rapid rates of transformation-excretion. For the same reason, the alkylating agent, spirohydantoin mustard might be a good drug for treating brain tumors by ia administration.

BBB OPENING BY HYPEROSMOTIC ARTERIAL INFUSION

Opening the blood-brain barrier by infusing a hyperosmotic solution into the carotid artery has been suggested by Rapoport (4) as a way of promoting the CNS uptake of drugs. In recent publications Rapoport and co-workers (5–7) have presented the first attempts to quantitate the magnitude of this opening. Our present studies on hyperosmotic opening are described in this section.

Normal adult male Sprague-Dawley rats, weighing 300–450 g, were used for this work. The procedure employed for opening the BBB was that of Rapoport et al (5), except that the BBB permeability to ^{14}C-α-aminoisobutyric acid (AIB), not ^{14}C-sucrose, was measured. Localized tissue activities of ^{14}C-AIB were determined by quantitative autoradiography (10,11). The blood-to-brain transfer constants, k_i, were calculated from the brain tissue and blood activity data and Ohno et al's equation 6 (12).

The k_i of AIB for normal brain tissue is $3–5 \times 10^{-6}$ sec^{-1}. Immediately after a 30-second infusion of the hypertonic solution, the permeability of AIB was markedly increased in many CNS areas. Table 8 shows the k_i values for several brain regions at various times after hyperosmotic opening. At 3 minutes (a measurement from $t = 0$ to $t = 6$ min after ending the ia infusion), the mean k_i values were 30–40 times greater than normal for most ipsilateral cortical grey matter areas and about 15-fold higher for corpus callosum; for the auditory cortex at this time, however, the mean k_i was only several fold larger than normal. By 25 minutes (a measurement made from $t = 20$ min to $t = 30$ min) all of the mean k_i values were at or approaching the control value. Normal k_i values were determined from 2 hours onward.

Not only did k_i vary with time and brain region but also within the same animal and region (the spatial resolution of this method of quantitative autoradiography is on the order of 100 μ). For example, in one animal from the 0–6-minute group, the following ranges of k_i values ($\times 10^{-6}$ sec^{-1}) were found: olfactory cortex, ipsilateral = 100–200, and contralateral = 20–100; parietal cortex, ipsilateral = 200–360 and contralateral, ≤ 5; and caudate nucleau, ipsilateral = 20–35, and contralateral ≤ 5.

TABLE 8.—Blood-to-brain transfer constants of ^{14}C-AIB (k_i) measured in the rat at various times after an intracarotid infusion of 1.6 M L(+) arabinose

Brain region	Side*	k_i ($10^6 \times sec^{-1}$) at various times†				
		3 min	10 min	25 min	2 hr	4 hr
Olfactory cortex	I	130 ± 35	25 ± 2	15 ± 4	5 ± 2	< 5
	C	85 ± 32	13 ± 5	13 ± 3	< 5	< 5
Parietal cortex	I	153 ± 23	37 ± 3	9 ± 2	< 5	7
	C	32 ± 18	< 5	< 5	< 5	< 5
Auditory cortex	I	20 ± 4	35 ± 5	18 ± 2	5 ± 1	< 5
	C	27 ± 9	17 ± 3	12 ± 2	< 5	< 5
Amygdala	I	148 ± 53	17 ± 4	13 ± 3	6 ± 1	< 5
	C	8 ± 2	< 5	< 5	8 ± 4	< 5
Corpus callosum	I	60 ± 10	8 ± 1	7 ± 2	< 5	< 5
	C	9 ± 4	< 5	< 5	< 5	< 5

*I = ipsilateral to the infused artery; C = contralateral.
†Mean ± SE; for each set N = 3; lower limit of resolution for this technique is $k_i = 5 \times 10^{-6}$ sec^{-1}

These findings indicate that transient opening of the BBB occurs in the rat after a 30-second unilateral intracarotid infusion of 1.6 M arabinose solution but that the degree of opening varies with time after administering the hyperosmotic infusate, CNS region, and even location within the region. Recently, Rapoport et al (6) have made a quantitative study in the rat of the duration of ia infusion and the concentration of the ia infusate and found that an infusion of at least 20 seconds with a 1.6–1.8 mM arabinose or mannitol solution is needed to effectively open the BBB. The longest period of ia infusion used in this study was 30 seconds; a longer infusion than this would probably produce a longer period of opening, but this cannot be said with certainty.

Rapoport et al (6) also report that hyperosmotic opening of the BBB leads to some brain uptake of fluid. A 5%–7% swelling of the half of the brain ipsilateral to the ia administration site was found 10 minutes after the infusion. This degree of cerebral edema persisted for at least 2 hours. After 1 day the water content of the ipsilateral hemisphere was still slightly elevated but approaching normal. Furthermore, these authors noted that the 30-second ia infusion procedure increased the serum osmolality of the rat from the normal level (300 mosmol) to 355–360 mosmol immediately after the completion of the infusion; at 10 and 50 minutes after the infusion, the serum osmolalities were 325 and 315 mosmol respectively. Changes in brain volume and serum osmolality would no doubt be more severe than these in long term (> 30 seconds) ia infusion of hypertonic solutions and could lead to untoward effects in patients.

Also, before trying to increase the uptake of a particular drug into brain tumors with such a technique, the following points should be considered. First, the agent must be cytotoxic for the primary or metastatic tumor cells growing within the CNS and be so within a reasonable tissue concentration range. If the drug is active against a particular type of cancer cell when located in non-neural tissue, it may not be effective in treating the CNS metastasis of this disease because of changes in the biology of this cell line caused by the brain's chemical environment. Second, the limitation in the substance's distribution within the CNS must be the BBB and not the membranes of the parenchymal cells. The intracarotid infusion appears to affect only the permeability of the BBB; the subsequent movement of the material from brain extracellular fluid into brain cells seems to occur in a normal manner. Third, the duration of BBB opening, the plasma and tissue concentration time courses of the agent, and the required exposure time of the tissue's site of action to the agent must be complementary. Therapeutic failure will occur, despite BBB opening, if the exposure time for complete pharmacological action is fairly long and the tissue's concentration of the material is inadequate during part of this period. Finally, some knowledge of the effects of the disease on local blood flow and capillary permeability is important. If blood flow to the tissue is very low, little of the hyperosmotic fluid will reach the appropriate site and the degree of "opening" will be greatly compromised; if brain capillary permeability has already been markedly increased by the lesion itself, the infusion will have little or no effect on BBB permeability and drug exchange.

In conclusion, extensive pharmacokinetic studies of hyperosmolar intracarotid infusions and pathophysiological examinations of various diseases are needed before this technique is used in the clinic as an adjunct to CNS chemotherapy.

GLOSSARY

IA = intra-arterial.
IV = intravenous.
X = the drug exposure or time integral, namely, the concentration-time product of the drug in the blood or tissue.
R_{di} = drug distribution advantage or the ratio of drug exposure obtained for a particular tissue after beginning an ia infusion to that obtained after beginning an iv infusion.
X_{IA} = the drug exposure or time integral found with an ia infusion.
X_{IV} = the drug exposure or time integral found with an iv infusion.
N_i = the number of drug molecules infused (assumed to be the same in all cases considered in this paper).
X_a = the integral obtained during the first passage of a substance through the arterial system following an ia infusion.
X_v = the integral obtained during the first passage of a substance through the arterial system following an iv infusion.
X_{ra} = the recirculation integral found with an ia infusion (note: $X_{IA} = X_a + X_{ra}$).
X_{rv} = the recirculation integral found with an iv infusion (note: $X_{IV} = X_v + X_{rv}$).
A = the total amount of administered drug.
CO = the cardiac output.
F_a = the rate of blood flow through the target artery.
α = the fraction of the cardiac output that flows through the target artery (F_a/CO).
ϕ_a = the ratio X_{ra}/A.
ϕ_v = the ratio X_{rv}/A.
p_l = the probability that the drug will be transformed or excreted in one passage through the lungs.
p_a = the probability that a molecule of drug will be lost from the body during a single passage through the entire circulatory system, including the lungs but excluding the target artery.
p_b = the probability that a drug molecule will be permanently lost from the circulation during a single passage through the target artery and tissue.
k_b = the target tissue's rate constant for drug transformation-excretion.
$\bar{t}$ = the mean transit time of a molecule through an organ's capillary system.
λ = the tissue:blood partition coefficient or the tissue distribution space of a substance.
E = the blood-to-tissue exchange or extraction fraction for a drug (a measure of capillary permeability).
$t_{1/2}$ = the half-time of drug transformation-excretion.
k_w = the rate constant for whole body transformation-excretion.
k_i = the blood-to-brain transfer constant for AIB.

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Osmotic Blood-Brain Barrier Disruption: Pharmacodynamic Studies in Dogs and a Clinical Phase I Trial in Patients with Malignant Brain Tumors[1]

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SUMMARY

Infusion of 25% mannitol in saline into the internal carotid artery of dogs disrupts the blood-brain barrier (BBB) in a controlled and reproducible manner (osmotic disruption), whereas mannitol infused through the common carotid artery produces variable disruption of the BBB. Compared to controls, infusion of methotrexate in dogs after osmotic disruption produced significantly higher drug levels in brain. We report very preliminary data on the effects of osmotic disruption with mannitol in five patients harboring malignant brain tumors.

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Chemotherapy has demonstrated little if any effectiveness in the treatment of primary and metastatic CNS lesions (1,2). Patients on "effective" therapy have developed brain metastasis concomitant with systemic tumor regression following the administration of adriamycin (3). Because the existence of an intact blood-brain barrier (BBB) at the proliferating edge of these tumors may be a prominent factor limiting drug delivery and effective chemotherapy (4,5), we began to evaluate disruption of the BBB by administration of hypertonic drugs as a possible method to increase drug delivery of anticancer agents to CNS tumors. Hypertonic infusions enabled such agents as microperoxidase, penicillin, Evans blue dye-labeled albumin, and various neurotransmitters to enter the central nervous system (6-8). Because initial animal studies verified augmentation of the methotrexate (MTX) levels in brain parenchyma following reversible osmotic BBB disruption (9), our investigation into reversible osmotic barrier disruption before administration of chemotherapeutic agents may be an enterprising approach to increasing drug delivery. This report is a synopsis of our initial animal studies and a phase I clinical trial in brain tumor patients of intra-arterial (ia) infusion of mannitol to reversibly disrupt the BBB.

ANIMAL STUDIES

A canine model analogous to the rat model of Rapoport (6) was used for our animal studies. All dogs used for invasive studies were anesthetized with barbiturate. The common carotid artery was initially chosen for mannitol infusion. The rate of infusion was established by electromagnetic determination of blood flow (60-500 ml/min in 25-kg mongrel dogs). Half of this rate (30-250 ml/min) was used to infuse 25% mannitol into the common carotid artery over 30 seconds. During infusion, the common carotid was clamped transiently (1-2 minutes) proximal to the site of infusion. Five to 15 minutes after the mannitol infusion MTX (100 mg in 30 ml saline solution) was infused over 15 minutes into the common carotid artery with a Harvard infusion pump. The intracarotid route of MTX infusion was chosen to attain higher brain levels of the drug with minimal total dose (10).

Evidence of barrier disruption was evaluated by two independent methods. In the first method, gross anatomical patterns of BBB disruption were evident by the degree of staining of the parenchyma after iv administration of Evans blue dye-albumin and were graded as follows: grade 0 = no staining; grade 1+ = just noticeable staining; grade 2+ = moderate

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blue staining; and grade 3+ = marked dark blue staining.

The second measure of BBB disruption was the distribution of MTX in the CNS after ia administration. The MTX levels of plasma, cerebrospinal fluid (CSF), and brain homogenates were quantitated by radioimmunoassay (9). Ipsilateral brain levels were in the range of 500–1000 ng of MTX/tissue; in the contralateral hemisphere, brain levels were 300–600 ng of MTX/g tissue. The therapeutic tissue level of MTX is generally considered to be about 300 ng/g. In control studies in which normal saline was infused in place of mannitol, ipsilateral brain levels of MTX were about 100 ng/g tissue (table 1).

Unfortunately, infusion of hypertonia mannitol through the common carotid artery gave somewhat variable BBB disruption as determined by transudation of intravenously (iv) administered Evans blue dye and variable brain levels of MTX. Consequently, burr-hole craniotomies were performed on several dogs, which provided direct visualization of the cortical surface of the brain; variable blanching of the ipsilateral cerebral cortex occurred during mannitol infusion.

A major technical advance was made by altering the site of infusion (9). When the distally located internal carotid artery was cannulated, 41 ml of 25% mannitol infused over 30 seconds completely and consistently blanched the cerebral cortex of 25-kg mongrel dogs. The staining of the brain parenchyma following iv Evans blue dye-infusion was also more pronounced and consistent than with common carotid artery infusion. With this method of infusion, brain levels of MTX 1 hour after normal saline averaged 1200 ng of MTX/g of tissue (N = 7). Dogs infused with mannitol and MTX through the internal carotid artery averaged 15,000 ng MTX/g of tissue (N = 16) (table 2). As reflected in the large standard errors of the mean, there is considerable variation in the degree of BBB disruption as evidenced by Evans blue-albumin staining and MTX tissue concentrations, which is attributable to the anatomical variation at the circle of Willis.

It is of particular note that CSF MTX levels did not always correlate with other measures of BBB disruption. While brain levels of MTX were markedly elevated in BBB disrupted animals as compared to control animals following internal carotid artery infusion, CSF levels often were quite similar. In addition, there was a poor correlation between brain/serum and CSF/serum ratios of MTX levels. Because CSF MTX levels did not always reflect brain levels, CSF sampling in clinical studies may not be as reliable as initially expected and may be inadequate as a parameter for pharmacologic monitoring of therapy.

We have used computerized tomography (CT) as a noninvasive monitor of BBB disruption (11). The CT provided a radiographic image of BBB disruption and provided a means of mapping the precise areas of the brain that were affected. CT technology also provided numerical values ("CT numbers") that correlated with the degree of BBB disruption and the ability of MTX to cross the BBB.

Conray 60 (5 ml/kg) mixed with Evans blue dye-albumin was administered iv. Immediately thereafter, 25% mannitol was infused into the internal carotid artery as described above. One hour after BBB disruption, the dog was sacrificed, the brain removed, and suspended in saline in a watertight container that was placed in the scanning aperture of an Artronix CT head-scanner. CT scan images revealed contrast enhancement in the regions of the brain parenchyma where the BBB had been disrupted.

We found an excellent correlation between regions stained with Evans blue dye-albumin and regions enhanced on CT scans. The attenuation differences between the normal regions of the brain and regions that had been disrupted and enhanced with contrast usually had a CT number difference of approximately 20 units, which corresponds to a 2% difference in attenuation relative to water and is easily detected on the CT scanner.

CLINICAL STUDIES

Because of the dismal clinical course of patients with primary (glioblastoma) and metastatic brain tumors and evidence that the failure of chemotherapy may be related to drug delivery, we initiated a phase I (toxicity) study of osmotic BBB disruption (12–15). To date, five patients with primary or metastatic CNS malignancies have been entered into this protocol. All patients had a histologic diagnosis and continued to receive conventional radiation therapy while on the study.

The study was approved by the institutional committee on human research and informed consent was obtained from each patient. Patients were carefully evaluated by unenhanced, enhanced, and double-dose-enhanced CT scans, angiography, radionuclide brain scan, and EEG.

The initial procedure of the protocol was placement of a burr hole in the temporoparietal region over the tumor-bearing hemisphere. Saline was infused through a femoral catheter into the internal carotid artery to determine the flow rate necessary to totally displace blood from the ipsilateral cerebral circulation. Using a flow rate of 6–9 ml/second, 25% mannitol was infused for 30 seconds.

A transient rise in intracranial pressure from 3–6 to 15–20 cm of H_2O was observed for 15–30 minutes. A Cushing response lasting 5–15 minutes was frequently documented by a dramatic rise in blood pressure and a marked drop in pulse. Except for EEG slowing during

TABLE 1.—Common carotid infusion of mannitol or saline* followed by common carotid methotrexate† infusion

Time from carotid infusion to tissue sampling	Saline infusion (ng methotrexate per gram of tissue)					Mannitol infusion (ng methotrexate per gram of tissue)				
	No. of dogs	Ipsilateral hemisphere *grey*	*white* matter	Contralateral hemisphere *grey*	*white* matter	No. of dogs	Ipsilateral hemisphere *grey*	*white* matter	Contralateral hemisphere *grey*	*white* matter
1 hour	18	110	52	160	88	17	940	720	990	740
						19	180	98	160	76
						20	660	370	98	54
						26	280	180	150	60
						$\bar{x}$	515	342	350	233
						SD‡	351	277	428	338
						SE§	175	138	214	169
3 hours	11	216		180		12	1800		720	
	13	99		81		16	360	340	250	250
	$\bar{x}$¶	158		131		31	200	79	46	26
	SD	83		70		33	205	28	55	59
						$\bar{x}$	641	149	268	112
						SD	776	167	316	121
						SE	388	965	158	699
6 hours	29	50	220	55		23	1770	780	198	130
						30	242	91	96	114
						32	47	29	47	28
						$\bar{x}$	686	300	114	91
						SD	944	417	77	55
						SE	546	241	45	32
26 hours						22	92	99	96	110
72 hours						21	19	15	19	16

*Mannitol or saline infusion volume = ⅓ carotid blood flow which was infused over 30 sec.
†Methotrexate (100 mg) infused over 15 minutes.
‡Standard deviation.
§Standard error of the mean.
¶Mean.

TABLE 2.—Internal carotid infusion of mannitol or saline followed by internal carotid methotrexate infusion

Dogs No.	Ipsilateral hemisphere			Contralateral hemisphere		
	Grey	White	Basal ganglion	Grey	White	Basal ganglion
Mannitol infusion (ng methotrexate/g tissue)						
39	5,800	17,000	7,200	1,900	640	1,000
45	10,500	11,000	1,500	240	110	170
50	28,000	20,000	9,200	2,800	710	830
52	25,000	19,000	2,700	1,900	710	1,500
54	2,000	1,000	400	440	110	130
55	90,000	33,000	1,800	580	350	780
57	1,300	350	270	150	78	130
58	8,200	4,500	920	220	78	84
59	10,000	19,000	7,100	6,200	1,600	240
60	3,400	2,200	2,000	1,300	300	220
61	2,700	2,900	4,900	2,800	260	710
62	18,000	7,300	3,200	1,200	260	710
63	18,000	28,000	8,200	4,100	2,700	1,100
64	6,000	4,000	1,100	3,100	1,100	300
67	20,000	18,000	12,000	10,000	4,200	540
$\bar{X}$†	16,593	12,483	4,166	2,462	880	593
±SD‡	±5,694	±2,671	±955	±693	±300	±111
Saline infusion (ng methotrexate/g tissue)*						
40	5,300	1,700	1,400	150	200	340
48	1,000	420	150	130	60	160
53	1,400	690	270	840	270	180
72	1,600	640	650	660	150	240
73	2,800	1,600	1,100	550	130	230
74	360	410	230	210	100	150
$\bar{X}$†	2,077	910	633	423	152	217
±SD‡	±723	±239	±211	±123	±30	±29

*Samples obtained 1 hour after methotrexate infusion.
†Mean.
‡Standard deviation.

the 30-second mannitol infusion, no other electrical abnormalities were observed by continuously monitoring the EEG. Neurological examination revealed no changes. Careful ophthalmologic evaluation did not reveal any changes in visual acuity or intraocular pressure. To date, no neurological complications have been observed after intracarotid mannitol infusion in patients followed for up to 2 months. BBB disruption has been documented in all patients by brain scan and/or "enhanced" CT scan.

In view of the lack of toxicity associated with these initial studies, patients are currently receiving intracarotid MTX 5 minutes after osmotic BBB disruption. It is hoped that this approach will provide substantial improvement in therapy that in the past has been only marginally successful.

Addendum

To date, MTX has been administered 33 times to six patients with brain tumors after BBB disruption with no permanent complications (16). No comment can be made at this time regarding the efficacy of this therapy.

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How Heterogeneous are Gliomas?[1]

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SUMMARY

This paper reviews studies in which cells from human gliomas grown in nude mice or passed serially in vitro showed heterogeneity as evaluated by morphological, biochemical, immunochemical, functional, and biological criteria.

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At this Conference and at the International Multidisciplinary Meeting on Brain Tumor Therapy held in June 1979, participants looked back over more than a decade at all aspects of basic and clinical research into the cause, diagnosis, and treatment of brain tumors. Although there has been much work, the only advances that have been almost universally incorporated into clinical care are the introduction of computerized axial tomography and the use of corticosteroids, which have reduced immediate postoperative mortality and morbidity caused by cerebral edema.

Our interpretation of clinical and experimental treatment studies shows that except in occasional individual hosts, gliomas are remarkedly refractory to surgery, and radiation, chemo-, or immunotherapy. Because of some success of the lipid-soluble nitrosoureas in glioma therapy and the lack of effect of most other drugs, there has been a great deal of emphasis on the postulate that a limiting factor in successful glioma chemotherapy is poor access of drug to all parts of gliomas. As discussed elsewhere in these proceedings (1), the blood-brain barrier is partially and randomly preserved in gliomas, and drug delivery may remain a significant problem, even though osmotic manipulation may allow opening of the barrier. Even if drug delivery problems are overcome, we are becoming convinced that heterogeneity or diversity in resistance to kill of glioma cell populations will be responsible for continuing resistance to current modes of chemo-, radiation, and immunotherapy.

On the other hand, recognition of the extent of cellular diversity within and among gliomas may allow rational design of new types of combination therapy individualized to patients. For example, each patient might require his or her own idiotypic combination of several drugs, or an individualized mixture of antigens for immunotherapy.

Both Shapiro's (2,3) and our group are focusing on heterogeneity to try to define its limits and establish its clinical significance. We are accumulating evidence for the heterogeneity of gliomas in cell lines and clones of human gliomas studied in culture and after serial passage of human gliomas in athymic mice (4–8).

At the morphological level, the cellular pleomorphism and heterogeneity of cell types in gliomas has been long recognized and is even embedded in the name "glioblastoma multiforme." In 15 permanent cell culture lines, clones, or sublines from anaplastic human gliomas, four main morphological patterns of cells have been observed: fibroblastic, fascicular, epithelioid, and "glial." These permanent lines were also heterogeneous with respect to all the other phenotypic and cell biological parameters we have studied. For example, although all the lines except two, derived from blacks, were homogeneous for glucose-6-phosphate dehydrogenase isoenzyme type B and reflect the caucasian genetic background of their donors, they were heterogeneous in most of their other characteristics (table 1). HLA phenotypes were idiotypic. All lines tested were aneuploid, but markers and chromosome modal numbers were different among lines. The percentage of cells responding morphologically to 1mM cyclic AMP added to the culture medium in the presence of theophylline ranged from 5% to 100%. CNPase (2′-3′ cyclic nucleotide 3′-phosphohydrolase) activity expressed as moles of 2′-AMP formed/hour/mg protein from homogenized cultures of the glioma lines varied from nondetectable levels to 12.78 ± 1.49 (table 1).

Other biochemical markers of neuroglia were variably expressed. No lines had detectable levels of

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TABLE 1.—Initial characterization of 15 permanent cell lines and sublines derived from human gliomas

Cell line*	Tumor of origin†	Highest passage level at Duke	G6PD‡ isoenzyme type	HLA phenotype
U-138 MG	Anaplastic glioma (M,48)	365	B	AW24,B27,BW51
U-410 MG	Glioblastoma multiforme (M,42)	125	B	A3,11,29,AW33,B2,12
U-105 MG	Glioblastoma multiforme (M,62)	320	B	A2,9
D-37 MG	Glioblastoma multiforme (F,61)	86	B	A1,2
U-118 MG	Anaplastic glioma (M,50)	566	B	A3,9,B8
U-343 MG	Anaplastic glioma (M,60)	236	B	A2,3,B7
U-251 MGsp	Glioblastoma multiforme (M,75)	214	B	A2,AW30,B8,1214,18
U-251 MG	Glioblastoma multiforme (M,75)	621	B	A1,2,3,AW24
D-32 MG	Gliosarcoma (M,66)	114	A	A10,29,AW33,B5,12
D-65 MG	Glioblastoma multiforme (F,65)	54	B	A2,AW31,BW15,17,22
A-172 MG	Glioblastoma multiforme (M,53)	155	B	A1,AW24,B7,8
D-18 MG	Glioblastoma multiforme (F,60)	196	B	A1,3,B8
D-54 MG	Mixed anaplastic glioma (F,39)	125	B	A1,B8
U-373 MG	Glioblastoma multiforme (M,50)	585	B	A1,B12
D-32 MGC2	Gliosarcoma (M,66)	79	A	A10,11,AW33,B5

*Italicized cultures were tumorigenic in athymic mice.
†Tumor classification according to WHO criteria by DDB, sex and age of patient follow in parentheses.
‡Glucose-6-phosphate dehydrogenase.

S-100 protein, but glial fibrillary acidic protein was detectable with an indirect immunofluorescence assay in three lines, although the distribution and intensity of fluorescent cells varied markedly among the lines. Fibronectin levels as measured by radioimmunoassay in culture supernatants and expressed in $\mu g/ml/10^5$ cells at confluence, ranged from nondetectable levels to 21.4. There was a general trend (not statistically significant because of the wide ranges in amounts of fibronectin expression) for highest levels of fibronectin to be found in lines of "fibroblastic" morphology; intermediate levels in those with fascicular and epithelioid patterns; and lowest in "glial" lines. Great variation was present in adult and fetal brain cell surface antigen expression. As measured in complement-dependent C^{14}-nicotinamide release assays with sequentially absorbed nonhuman primate antiserum raised against four of the individual cell lines and one human glioblastoma, there was complete individuality among the lines in expression of idiotypic and adult or fetal brain-shared surface antigens.

In addition to heterogeneity in biochemical and immunochemical properties, all of the lines showed marked variation in their cell biological properties. Percent colony formation ranged from a low of 0.3% for U-343 MG to a high of 21.0% for U-251 MG. Absolute plating efficiency ranged from 1.6% to 16.3%; saturation densities from 0.93 to 6.12×10^6 cells; and population doubling times from 25.9 to 58.3 hours.

Heterogeneity in phenotypic and cell biologic characteristics measured in vitro can only be used as indirect evidence to postulate or predict heterogeneity of chemotherapeutic response in vivo. In order to study in vivo response of the permanent lines to chemotherapeutic agents, we have established model systems for glioma chemotherapy in athymic "nude" mice (nu/nu Balb/c genotype).

Six of our permanent lines (table 1) were tumorigenic when approximately 10^6 cells were injected sc . Only four of the lines (U-251 MG, U-118 MG, U-373 IMG, and D-54 MG) grew progressively and could be serially passed. Three of the four lines (U-251 MG, U-118 MG, and D54 MG) grew as solid tumors, while the fourth was cystic and hemorrhagic. There was a linear correlation between caliper measured volume and weight of tumors over a wide range of tumor weights (1–14). In early passage, the tumors were morphologically distinct from one another, and had homogeneous patterns somewhat similar to the morphological patterns of cells at confluence in culture. With serial passage, there was less difference among the tumors and assumption of more anaplastic, poorly differentiated morphological patterns, highly reminiscent of a poorly differentiated sarcoma in the case of U-251 MG. Initially, both among and within the lines there was great variation in latency and volume doubling times. With serial passage, biological stability was attained, usually between the third and fifth passage levels.

Once biological stability was achieved, we measured response to chemotherapy at a constant dose of 25 mg/kg of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) given ip 7 days after transplantation to animals with sc tumors. Percent reduction in tumor mass was compared for each line in ten BCNU-treated to ten vehicle-treated controls and percent differences calculated. Statistically significant differences in BCNU-treated animals were observed as early as 7 days after BCNU treatment. Maximum differences were > 80% reduc-

tion in tumor volume for U-251 MG and U-118 MG, and > 60% reduction for D-54 MG. Expressed as difference in days for treated versus control animal tumors to reach a mass of 1.5g, the results were 15 days for U-251 MG, 17 days for U-118 MG, and 7 days for D-54 MG.

We have also studied single cell-derived clonal populations from the same tumor to determine if there is heterogeneity within a permanent cell line as well as among the different lines. Seven clonal populations derived by isolation of single cells by end point dilution plating were studied from parent line D-54 MG. Whereas the parent line had a "glial" morphologic pattern, only five of the seven clones were "glial," with one mixed and the other epithelioid. CNPase, GFA, and surface antigen expression also varied among the clones. Three of the clones were tested for tumorigenicity in athymic mice, and like the parent line, all were tumorigenic and could be serially transplanted. Biological stability was achieved by serial passage for two lines; nevertheless, their volume doubling times were quite distinct. The third clone has not yet achieved biological stability in its growth properties and is still being serially passed. We conclude that phenotypic and biological diversity is present among clones from a permanent glioma cell line.

It is theoretically possible that the diversity of permanent cell lines from human gliomas might be an artifact of culture conditions and prolonged serial passage in vitro. Moreover, the number of cell divisions that may have occurred in serial passage of permanent lines may be much greater than would have taken place in a patient at the time of diagnosis, allowing divergence in vitro that might not be present earlier in the natural history of a glioma.

Therefore, we are also studying gliomas from surgical biopsy material directly transplanted into nude mice. Approximately two-thirds of the 26 human central nervous system tumors we have implanted sc or intracerebrally into athymic mice have survived heterotransplantation (table 2). Progressive growth, defined by sustained increase in mass and the ability to be serially passed, has been observed in 10 of 14 anaplastic gliomas and gliobalastomas. The morphological heterogeneity of gliomas, so striking in surgical biopsy specimens as shown in figure 1, is not maintained in transplantation in nude mice, but is replaced by a morphologically homogeneous pattern.

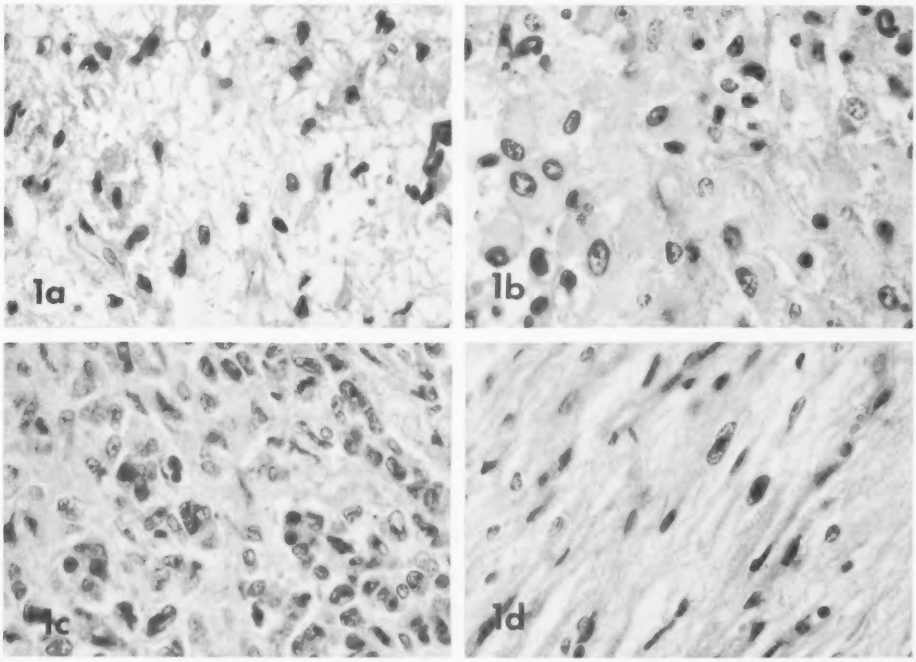

FIGURE 1.—Human glioblastoma multiforme 91 is remarkably heterogeneous, composed of *(a)* fibrillary cells with microcystic change, *(b)* polygonal cells with abundant cytoplasm, *(c)* small cells with a high nuclear to cytoplastic ratio and *(d)* elongated bipolar cells (hematoxylin and eosin, × 400.)

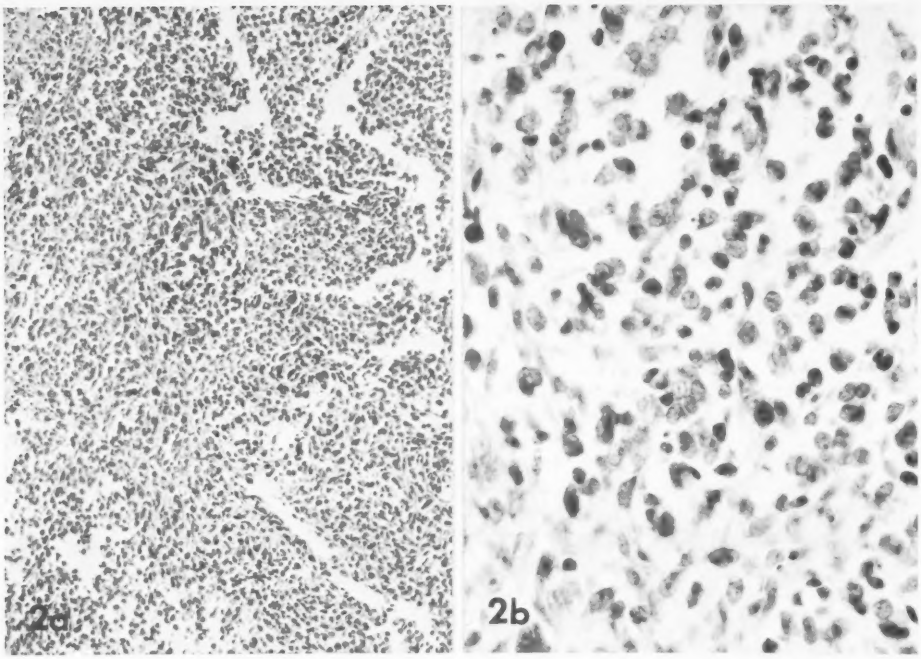

FIGURE 2.—In contrast, this same human glioblastoma 91 grown sc in the nude mouse contains a single, homogeneous population that on higher magnification is composed of small cells with a high nuclear to cytoplasmic ratio. *(a)* Hematoxylin and eosin, × 100; *(b)* hematoxylin and eosin, × 400.)

TABLE 2.—Heterotransplantation of human nervous system tumors in athymic mice

Tumor type	Number of takes/ number of attempts
Anaplastic gliomas*	12/14
Astrocytomas	0/2
Ependymomas	2/3
Craniopharyngiomas	2/3
Metastatic adenocarcinomas	3/4
Total	19/26

*Only the anaplastic gliomas were observed for progressive growth, defined by the ability to be transplanted serially, and progressively enlarge. By that definition 10/14 of the anaplastic gliomas grew progressively.

The three glioma lines—9L, I32, and I75—that we have studied in detail have become biologically stable by the third passage. Nevertheless, their volume doubling times have remained individually distinct at 5.5, 9.7, and 3.6 days respectively. These lines are now being tested to determine if heterogeneity exists in their response to chemotherapeutic agents.

The morphological heterogeneity responsible for naming the most malignant glioma "glioblastoma multiforme" is characteristic of most gliomas studied. All show morphological, biochemical, immunochemical, functional, and biological heterogeneity in vitro and in tumors serially passed in vivo in nude mice. We believe that the subcutaneous growth of human gliomas in nude mice is an excellent system in which the extent of heterogeneity among glioma patients and among cell populations of individual gliomas can be explored because there is a high take rate of human glioma cells in nude mice and drug access to sc tumors is less of a problem than for intracerebral tumors.

It is possible that there is a limit to heterogeneity among and within gliomas. If those limits can be defined, and idiotypic patterns of heterogeneity recognized, then more rational and effective combinations of therapies can be devised.

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Clonogenicity and Growth of Experimental Tumors in Relation to Developing Resistance and Therapeutic Failure[1]

Daniel P. Griswold,[2,*] Thomas H. Corbett,[2] and Frank M. Schabel, Jr.[2,3]

SUMMARY

This paper briefly reviews the results of in vivo experiments with animal models that show the development of tumor cell resistance to chemotherapeutic agents. Implications for clinical treatment of solid tumors are discussed.

[Cancer Treat Rep 65 (Suppl 2):51–54, 1981]

As progress in the treatment of cancer advances, it is common to see many initial responders to chemotherapy subsequently fail, even in the face of continuing treatment. These observations have been made in leukemias and solid tumors of humans and in a wide variety of animal experimental tumors. Treatment failures are marked by the resumption of tumor growth during continuing treatment that initially showed response with marked regression (1).

Failure to obtain a response to chemotherapy or failure after cessation of chemotherapy considered to be optimal has been attributed to a number of phenomena, including pharmacologic and anatomic sanctuaries, tumor cell hypoxia, a G_0 kinetic state, and repair of sublethal or potentially lethal damage. Data we have gathered from experiments with murine tumor models suggest that the selection and overgrowth of tumor cells resistant to specific drugs is a major reason for treatment failure that follows an initial early response (1).

BACKGROUND

The development of resistance of a cell population to an exogenous agent is not a newly recognized phenomenon. For decades, resistance was a problem faced by bacteriologists, and even before the antibiotic era bacterial resistance to viral bacteriophages was well recognized. In the 1920s two mechanisms were thought to be responsible for the development of (or mutation to) resistance. One proposed that resistance resulted from exposure to the agent to which resistance developed, while the other proposed that mutation to resistance was spontaneous, occurring in the absence of an inciting agent. Burnet (2) favored the spontaneous view and his data tend to support that position. However, it was not until 1943 that spontaneous mutation to resistance was conclusively demonstrated. Using a fluctuation test, Luria and Delbruck (3) demonstrated the spontaneous mutation to resistance of *Eschericia coli* B to a bacterial virus. In 1952, Law (4), also using a fluctuation test, confirmed these observations when he found development of resistance to amethopterin by the murine leukemia L1210. Law's data not only demonstrated the spontaneity of mutation to resistance, but also demonstrated the randomness of the event. More recent data suggest that although mutation is a random event, it is random within limitation. The rate of mutation to resistance among some murine tumor cell populations is dependent on the class of chemotherapeutic agents to which they become resistant, being highest with mitotic inhibitors, lowest with highly reactive agents such as alkylating agents, and intermediate with antimetabolites (1).

The clinical course of patients harboring advanced solid tumors who are treated with chemotherapeutic agents, and the results of surgery and chemotherapy trials, suggest that resistance is an obstacle to successful chemotherapy.

Shown in figure 1 are part of the data from an experiment in which each of 20 hamsters bearing an advanced (ca 1.0 g) plasmacytoma were treated with a 90 mg/kg dose of cyclophosphamide six times at 15-day intervals. Complete tumor regression was observed in

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[3]The authors are indebted to Dr Howard E Skipper, who taught them to look at the data.

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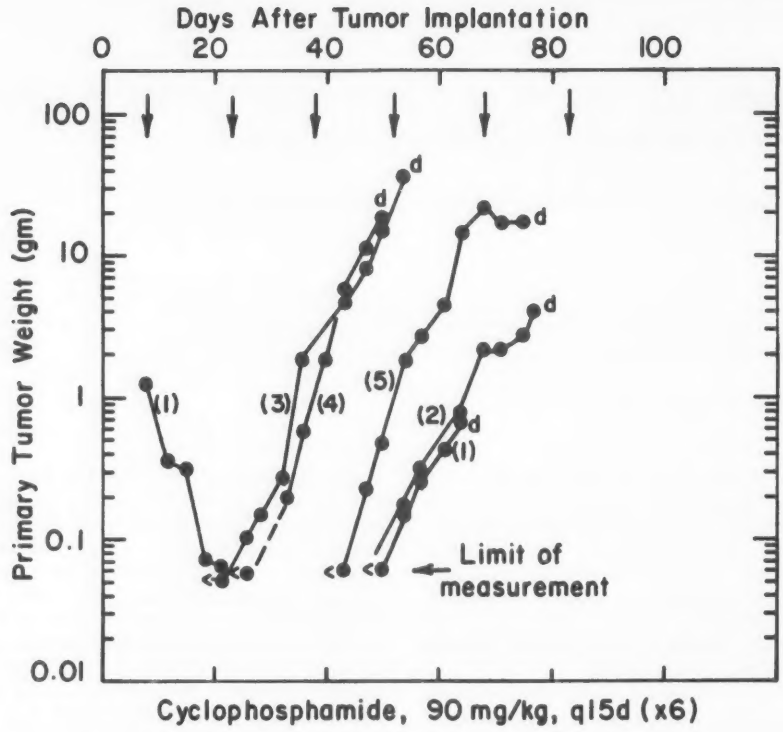

FIGURE 1.—Complete regression and recurrence of sc-implanted plasmacytoma in hamsters during continued and undiminished treatment with cyclophosphamide. The regression curve of only tumor 1 is shown; the rate of regression of the other tumors was similar.

65% of the hamsters. Three tumors never recurred, and the hosts were considered cured. But five of the tumors did recur, and did so in the face of continuing treatment. Moreover, when recurring tumors from similar experiments were transplanted to healthy hamsters that were then treated with cyclophosphamide, the tumors were solidly resistant (5). While the measurable recurrence during continuing treatment of tumors that initially regressed under the same treatment is highly suggestive of developing drug resistance, that deduction is less easily made solely from animal or patient survival data.

Evidence of resistance is suggested in the results of the surgery/chemotherapy trial shown in figure 2. sc implanted No. 26 colon tumors (0.5–1.0 gm) were surgically removed from 30 mice. Four days later, 15 of the mice were treated with a single dose of a combination of mitomycin-C and 5-FU; the other 15 mice were not treated. Comparison of the survival curves of those two groups shows an increased median survival time of the drug-treated surgery group. The overall survival rate for the two groups was not different — there were 13% (2/15) tumor-free survivors in both groups. Thus the apparent initial effect was seemingly lost. Analyses (6) that consider the distribution of mice with different tumor burdens at the end of surgery or treatment suggest that treatment causes a shift towards the zero (10^{-1}) cell compartment. The percentage of tumor-free animals (cures) after treatment would then be a function of the fractional tumor cell kill and the number of mice whose tumor cell burdens were not greater than that tumor cell kill capability. Such would be the case in a situation where the surgery-only group and the surgery-plus-chemotherapy group had similar normal distributions before treatment and where some mice survived in the surgery-only group. Thus a displaced survival curve (indicative of tumor cell kill) for the surgery-plus-chemotherapy group would lead one to expect an increased percentage of tumor-free survivors in that group. The failure to see an increased cure rate in this example (fig 2) suggests that a larger-than-expected number of mice had small numbers of surviving tumor cells after treatment and further suggests the presence

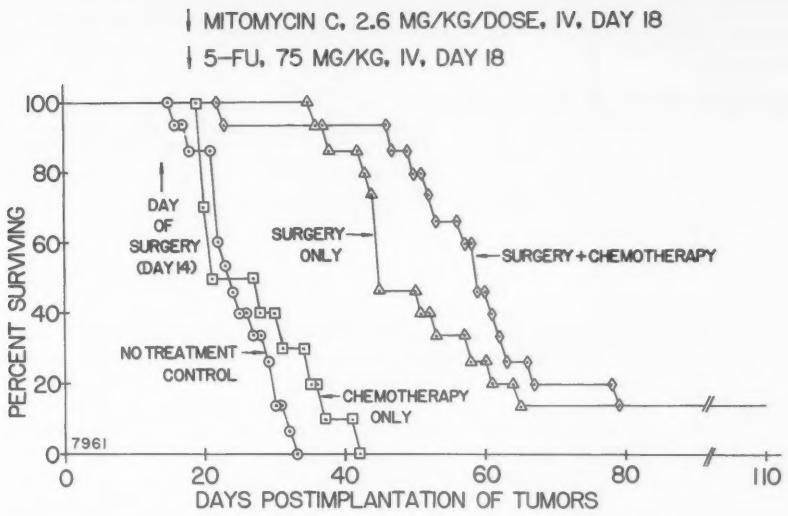

FIGURE 2.—Survival of mice with sc-implanted colon tumor 26 after surgical tumor removal with and without mitomycin-C and 5-FU treatment. Tumors were 0.5–1.0 at surgery.

of a mechanism that prevents drug kill of those last few tumor cells.[4] In this example it is also possible that the displaced survival curve resulted not from a drug kill-inhibiting mechanism but was an artifact that resulted from dissimilar distributions of mice with different tumor burdens in the two treatment arms. This is suggested by the lack of response of the group treated with chemotherapy alone, although that occurrence would not necessarily negate the possibility of response of the surgery-chemotherapy group, since the latter group had a much smaller tumor burden. In any event, the basis for failure to obtain a greater cure rate, even though some response to chemotherapy was observed, is not easily explained from survival data. Nevertheless, drug resistance remains a serious consideration.

MECHANISM OF SELECTION OF DRUG-RESISTANT TUMOR CELL POPULATIONS

If mutation to resistance is spontaneous, as all presently available data indicate, then it is likely that mutation to resistance has occurred by the time a tumor becomes clinically detectable. The smallest clinically detectable tumor in humans is estimated to contain about 10^9 cells (7), a greater tumor burden than in resistant mouse tumor models (1). Once mutation has occurred, the selection of a resistant tumor cell population will result from the death of drug-sensitive cells. This can be demonstrated from the data in figure 3. When mice bearing sc-implanted B16 melanoma were treated with a single 36 mg/kg dose of MeCCNU, compared to untreated controls a marked delay in growth indicated a significant tumor cell kill. Subsequent passage of drug-treated tumors to untreated and to-be-treated groups (four passages and four treatments) produced a gradual loss in tumor growth delay. After three to four passages and treatment, all antitumor effect of MeCCNU was lost. The rate of selection of a drug-resistant tumor cell population is dependent on the rate of growth of the resistant population and the rate of cell kill of the sensitive population.

IMPLICATIONS FOR CHEMOTHERAPY REGIMEN DESIGN

Using the mouse leukemia L1210 model, Skipper, Schabel, and Lloyd (8) have clearly shown that an otherwise effective drug treatment regimen will lose its capability to produce an increasing disease-free interval, duration of remission, or survival time when a mixed population of sensitive and resistant tumor cells reaches a 1:1 ratio. On that basis, it is evident that a change in therapeutic agents is necessary to provide additional improvement in duration of survival. Chemotherapy trials with murine leukemias (1,8) have indeed shown that a change of agent administered at

[4]Although a number of factors such as cell repair, pharmacologic sanctuary, and kinetic state might account for the failure of drug therapy, specific drug resistance must also be considered as a contributory mechanism.

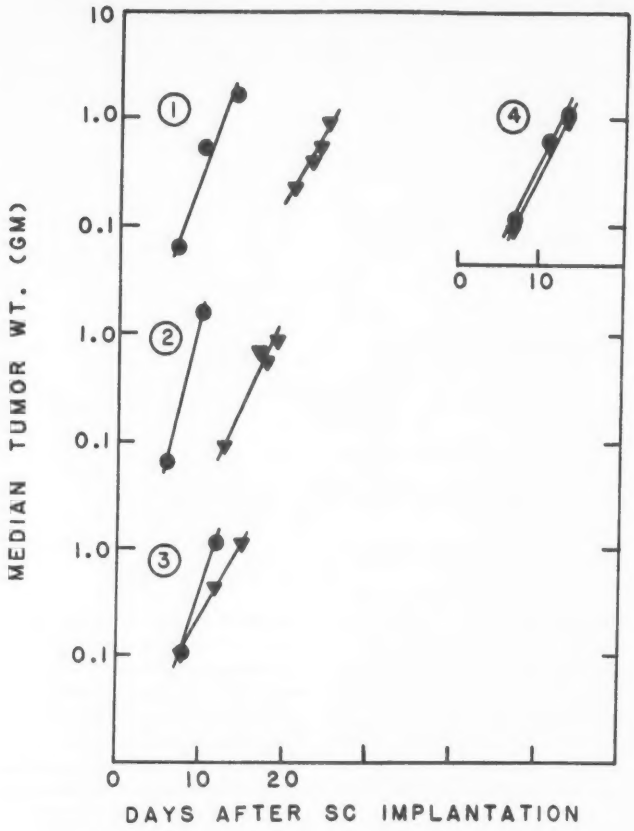

FIGURE 3.—History of the development of a MeCCNU-resistant line of B16 melanoma. Tumors were implanted sc. Circled numbers indicate number of passages from sensitive line. • = untreated control from previous MeCCNU-treated group. ▲ = MeCCNU, 36 mg/kg, Day 2 only from previous MeCCNU-treated group.

the approximate nadir to drugs that are effective against the resistant portion of the population can result in increased survival time and even cure.

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Heterogeneous Response to Chemotherapy of Human Gliomas Grown in Nude Mice and as Clones In Vitro[1]

W. R. Shapiro,[2,*] W. A. Yung,[2] G. A. Basler[2], and J. R. Shapiro[2]

SUMMARY

Our experience with the chemotherapy of immunodeficient nude mice bearing implanted intracerebral (ic) or subcutaneous (sc) human malignant gliomas has demonstrated heterogeneous responses to the same agents among tumors from different patients. In these experiments, tumor specimens obtained at craniotomy were dissociated and implanted directly, both ic and sc, into Swiss nu/nu athymic mice. Tumor implants readily grew after long initial delay times (20–150 days) that became shorter on serial transplantation. A comparison of the potential of BCNU and procarbazine to inhibit sc tumor growth and prolong survival of ic-tumor-bearing mice was made for tumors from six patients. One line was very sensitive to BCNU and insensitive to procarbazine; two lines were moderately sensitive to both drugs; one was minimally sensitive to both drugs; and one was minimally sensitive to BCNU and very sensitive to procarbazine. These results were consistent with the hypothesis that tumors might be comprised of several populations of cells, some of which adapted better to growth in mice. To test this hypothesis, human gliomas were obtained after craniotomy, dissociated into single cells that were then karyotyped (the "reference set"), and grown in monolayer tissue culture as clones. Clones were characterized by cell growth kinetics, GFAP, and chemosensitivity to BCNU and cis-platinum, and were rekaryotyped at regular intervals for comparison with the reference set and to test for homogeneity. The reference set disclosed extensive heterogeneity, with the number of chromosomes per cell varying from hypodiploid to hypertertraploid. Individual karyotypes demonstrated a variety of chromosomal abnormalities. Unstable clones often outnumbered stable clones, and were constantly undergoing chromosomal rearrangement. Heterogeneity was found in growth kinetic parameters and in response to different agents. These studies imply that tumor heterogeneity must be a consideration in the design of chemotherapy of brain tumors. The methods used here offer the possibility of studying clonal cell lines identifiably part of the patient's tumor.

[Cancer Treat Rep 65 (Suppl 2):55–59, 1981]

Experience with the chemotherapy of both leukemia and solid tumors has suggested that combination drug treatments generally are more effective than single-agent chemotherapy. This is best illustrated by the treatment of acute lymphatic leukemia in children (1) and Hodgkin's disease in young adults (2). Moreover, multimodality therapy and combination chemotherapy are now being utilized in a variety of clinical treatments for lung cancer, breast cancer, and the germinomas. The rationale for combination chemotherapy is that human cancers contain populations of cells with varying degrees of sensitivity to individual agents. When a single agent is used, only some cell populations are killed, which permits overgrowth by more resistant cell lines within the tumors.

That human cancers have heterogeneous cell populations of varying chemosensitivity is not easy to prove, especially for human malignant gliomas in which sampling error is a problem. Nevertheless, recent

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evidence from this laboratory suggests that not only do different patients' gliomas vary in sensitivity to similar agents, but that a single tumor contains populations of cells with varying sensitivity to the same chemotherapeutic agent. This report reviews briefly our experience with human brain tumors grown in the nude mouse and tested with chemotherapeutic agents and our experience with the heterogeneity of individual cell lines cloned from human glioblastomas.

MATERIALS AND METHODS

Nude Mouse Model

In these experiments, we used inbred Swiss athymic nude mice (nu/nu) housed separately from other mice. Fresh human glioma tissue was removed from patients at operation and was separated into portions that were implanted directly into nude mice or explanted into monolayer tissue cultures. For intracerebral (ic) or subcutaneous (sc) animal implantation, tumor tissue was immediately placed in minimal essential media (MEM) and distributed as cell suspensions or small tumor fragments (3). For ic inoculation, $1–5 \times 10_5$ cells in 0.01 ml of MEM were injected percutaneously into the right cerebral hemisphere. For sc inoculations, $1–5 \times 10^7$ cells in up to 0.1 ml of MEM were injected percutaneously into the flank. Both ic and sc inoculations were performed in a number of experiments, but not for all patients' tumors.

Nineteen of the initial 25 sc transplantations were successful at least once, as were 11 of 21 ic transplantations. Part of the failure was related to an epidemic of murine hepatitis in the colony. Tumors grew from 20–150 days after initial transplantation, but grew at a faster rate on subsequent serial transplantation. Most of the chemotherapy trials were conducted using tumor carried by sc passage. However, in several cases, cells grown in tissue culture were transplanted into mice, and in three cases tumor tissue excised from a patient was implanted directly into mice.

For the chemotherapy studies, we compared tumor responses after administration of either 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) or procarbazine hydrochloride. The BCNU was given at a dose of 30 mg/kg once (occasionally twice) intravenously (iv). The LD_{10} for this drug in C57 B1/6 mice is approximately 34 mg/kg and in the nu/nu mice approximately 40 mg/kg (4). The procarbazine was given iv at a dose of 100 mg/kg twice a week for four doses (in one experiment it was given at 300 mg/kg twice a week for two doses). The LD_{10} for this drug in C57 B1/6 mice is difficult to determine but has not been lethal at 500 mg/kg for a single dose. Generally, the drug was administered when 40%–60% of the mice had splitting skull sutures or had developed signs of neurological deficit. For tumor line JM, treatment was begun on Day 161 after initial transplantation and for tumor line JC it was begun on Day 140 after primary transplantation.

In Vitro Clones of Human Gliomas

For the cloning experiments, freshly resected glioma tissue was mechanically dissociated into single cells using Waymouth MAB 87/3 with 20% fetal calf serum as described (5). These cells were divided for chromosome preparations and cell culture. Q- and G-banded karyotypes were made from suspension cultures 6–24 hours after surgical resection and from short-term monolayer cultures 48 hours and 72 hours after surgical resection. Metaphase karyotypes from the three time intervals were combined to compose the reference set of karyotypes.

The remaining portion of the single cell preparation grew into colonies of 8–24 cells each. The colonies were characterized by karyotypic analysis, S100 protein, glial fibrillary acidic protein (GFAP), cell growth kinetics, chemosensitivity, and the ability for ic reimplantation into mice. The karyotypic analysis was performed at passages 2, 4, 6, 8, and 12 to determine the clone's homogeneity. We used the immunoperoxidase staining method to determine the presence of GFAP (6).

Chemosensitivity testing was performed by using a microcytotoxicity assay. One hundred to 150 cells were plated into each well of a Teresaki microtiter plate. After 48 hours of incubation, cells were exposed to drug solution at various doses for 1 hour. After another 24 hours of incubation, the cells were washed, fixed, and stained with Giemsa. The stained cells were then counted with a standard transmission microscope.

RESULTS

Tumors Grown in Nude Mice

The overall results of the chemotherapy program are shown in table 1. For tumor cell lines from patients 1 and 2 we have chosen the best results, some of which were reported previously (7). As can be seen, there were significant intra- and intertumor differences to both drugs, which ranged from a response to BCNU only to a response to procarbazine only. For the ic experiments, the percent increase in life span of the treated over the control group (ILS) is shown directly, while for the sc experiments the results are given semiquantitatively by the number of plus marks.

Human Glioma Cells Cloned In Vitro

Figure 1 shows the frequency distribution of the number of chromosomes found in the dissociated

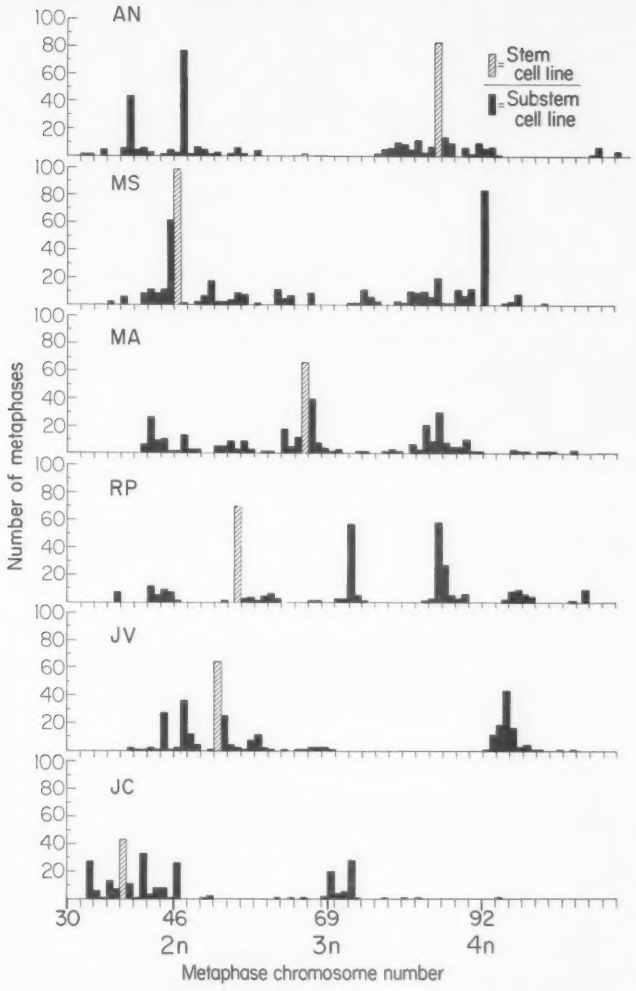

FIGURE 1.—Distribution of chromosome numbers in six human glioblastoma multiforme tumors. Cytogenetic analysis of 200 or more metaphases were analyzed to determine the number of cell populations present at the time of surgical resection. The stem cell line is the largest population of cells having the same chromosome number while the remaining populations constitute the substem lines.

tumor cells from the first six patients studied. The "stem cell line," defined by Levan et al (8) as the largest population of cells having the same number of chromosomes, was defined in this series as one represented by at least five identical karyotypes. The histogram demonstrates extensive heterogeneity with karyotypic deviations extending from the hypodiploid to the hypertetraploid chromosome range.

Table 2 lists characteristics of six of the eight clones derived from a patient's tumor. Most testing was done on the first 12 passages, although in some cases doubling times were performed on both early and late passages. Karyotypes varied from hypodiploid to near-diploid and near-tetraploid lines. As defined by one of us (Shapiro, JR, manuscript in preparation), RPC-10 was an unstable clone producing many new variant cell

Table 1.—Chemotherapy of human brain tumors implanted either intracerebrally or subcutaneously in nude mice

		BCNU					Procarbazine				
		Intracerebral			Subcutaneous		Intracerebral			Subcutaneous	
Patient	Source*	Dose (mg/kg)	T/C†	ILS‡ (%)	Dose (mg/kg)	Result	Dose (mg/kg)	T/C†	ILS‡ (%)	Dose (mg/kg)	Result
1	SC	30 × 2¶	85/33	158	30 × 2¶	+++	300 × 2¶	32/33	0	300 × 2¶	0
2n§	SC	30 × 1	61/44.5	37	30 × 1	+	100 × 4**	92/44.5	107	100 × 4**	++
2c§	TC	30 × 1	37/30	23	—	-	100 × 4**	60/30	100	—	-
3	SC	30 × 1	89.5/74	21	—	-	100 × 4**	76/74	0	—	-
4	P	30 × 1	233/167	40	—	-	100 × 4**	206.5/167	24	—	-
5	P	30 × 1	43.5/38	14.5	30 × 1	++	100 × 4**	49/38	29	0.100 × 4**	++
6	P	30 × 1	224/181	24	—	-	—	—	—		

*Source: SC = transplant from nu/nu sc tumor; TC = transplant from tissue culture; P = primary explant from patient.
†T = median day of death of treated group; C = median day of death of control group.
‡ILS = increased life span as percent greater than control.
§2n = tumor carried in nude mouse; 2c = tumor carried in tissue culture.
¶Dose weekly.
**Dose twice weekly.

Table 2.—Characteristics of some RP clones

	RPC-1	RPC-2	RPC-3′	RPC-4	RPC-10	RPC-12
Karyotype, passage 8	45XX,−11	37XX, del (lp), −3,−5,−7,−8,−9, −9,−15,−16,−16	47XX,+8 del (10p)*	89XXXX,† −8,−9,−15	83XXXX,† −3,−5,−6,−12,−13, −14,−18,−18,−21	46,XX t(2:3)
Chromosome range	45–48	37–39	40–97	89–92	69–92‡	46–47
Morphology	Astrocytic with processes	Squamous-like	Squamous-like	Fibroblast-like	Fibroblast-like	Fibroblast-like
Doubling time in culture (hours)	56	120(18)§ 168(10)	132(6)	84(8) 96(5) 96(13)	72(13)	72(12)
GFAP	±	±	+	+	+	±
BCNU ED_{50}, μg/ml	45	74	47	81	74	43
Cis-Pt ED_{50}, μg/ml	210	135	260	104	200	340

*RPC-3′ has a second mode at 86XXXX,−1,−6,−9,−11,−16,−18 (4n tetraploid cell).
†Chromosomes missing from a 4n tetraploid cell.
‡RPC-10 has a second and third mode at 85XXXX,−1,−4,−8,−9,−16,−21,−X; 88XXXX,−3,−9,−18,−18.
§Figure in parentheses = passage number.

types, while others remained stable through passage 12. Morphologically, the cloned lines varied from astrocytic with multiple processes to squamous-like and fibroblast-like. Doubling times in this series ranged from a low of 56 hours for RPC-1 to a high of 168 hours for RPC-2. On staining for GFAP, three clones were strongly positive, while three were only weakly positive.

The ED_{50} values for the microcytotoxicity assay performed with BCNU and cis-platinum are shown in table 2. RPC-12, a relatively fast-growing line, is highly sensitive to BCNU but almost insensitive to cis-platinum, while RPC-4 is quite sensitive to cis-platinum but much less sensitive to BCNU. The ED_{50} doses for this particular cytotoxicity assay are likely to be higher than those obtainable in patients, but the assay permits a rapid comparison among the clones to the two agents tested.

DISCUSSION

These in vitro investigations demonstrate that at the time of surgical resection human brain tumors contain heterogeneous populations of cells with different karyotypes. Each of our tumors had 11–21-cell populations. Most of the cells are unstable and capable of generating new variant cell types. The cell populations were heterogeneous in morphology, cell growth kinetics, and most importantly, in response to chemotherapeutic agents in vitro. It appears that the response of tumor cells to drugs in vitro or in vivo in mice will parallel the response to drugs in situ.

This study implies several conditions. Response to different drugs means that single agent chemotherapy of human brain tumors is unlikely to produce cures. The fact that stem cells can quickly give rise to new variants means that *all* stem cells must be eradicated quickly. Similar approaches may correlate chemosensitivity, karyotype, and the other phenotypic markers.

These methods permit us to use in vitro techniques to study the dynamic behavior of tumor growth with a lesser risk of introducing tissue culture artifacts than has been possible in the past. By isolating and expanding only cells identified in the reference set of karyotypes, only cell populations present in the patient's tumor, and not chromosomal variants arising in cell culture, can be studied. Single cell clones derived from a patient's tumor can be examined for surface proteins, mechanisms of drug resistance, cell-cell interactions, or a multitude of other characteristics associated with the cancer cell.

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Potentials and Limitations of a Clonogenic Cell Assay for Human Brain Tumors[1]

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SUMMARY

Single cells from biopsies of ten malignant gliomas were treated with 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) in vitro, and tumor cell survival was compared to patient response to nitrosourea therapy. The limitations of the methods and their interpretations and future prospects for clonogenic cell analyses are discussed.

[Cancer Treat Rep 65 (Suppl 2):61–66, 1981]

An assay for clonogenic cells of human brain tumors has been developed (1). Although glioblastoma multiforme had the greatest cloning capacity (colony-forming efficiency, or CFE) found among glial neoplasms, there did not appear to be any strong correlation between a tumor's cloning capacity in vitro and the patient's clinical course. Analysis of the chemotherapeutic sensitivity of clonogenic cells derived from biopsy specimens of tumor may help determine if a tumor may be responsive or resistant to chemotherapy.

Cell sensitivity testing entails a number of potential problems such as selection of a representative cell population, the inherently low CFE, differences between in vitro and in situ treatment, and the development of in situ resistance. To determine the feasibility of the methods required for, and the applicability of, such testing to the treatment of human tumors it is necessary to correlate in vitro results and patient response.

This report presents the preliminary results of our comparison of tumor cell survival in vitro and patient response after treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU). The limitations of the methods and analyses are discussed.

MATERIALS AND METHODS

Tumor Cell Analysis

The procedure for obtaining monolayer clonal outgrowths of cells derived from human brain tumor specimens has been described (2). Biopsies were received fresh from the operating room and within 2 hours were minced to < 1 mm^3 pieces and treated with 0.25% trypsin (Gibco) with 2 mg% EDTA at 37°C for 10 minutes. After filtration to remove nondigested particles, the single cell suspension was aliquoted into 60-mm plastic petri dishes at various dilutions. Plates were incubated at 37°C in a 5% CO_2-95% air humidified atmosphere for 4 weeks in an enriched medium containing 30% fetal calf serum and 5×10^4 heavily irradiated feeder cells. A collection of more than 25 cells of similar morphology was considered a colony that had originated from a single "clonogenic" tumor cell from the initial biopsy specimen.

Single cells were treated in suspension with various concentrations of BCNU for 2 hours at 37°C with continuous stirring, then washed and plated for cell survival. Colonies developed after incubation for 4 weeks under conditions optimized for in vitro growth (2).

Patient Population and Analysis

The clinical histories of ten patients harboring malignant gliomas whose specimens were analyzed are summarized in table 1. Five patients received a course of BCNU in the immediate postoperative period. Five patients were irradiated first with concurrent hydroxyurea administration, and subsequently received either BCNU or CCNU alone or as part of a combination drug regimen (patient 6). Cellular sensitivity and tumor response to BCNU and CCNU were considered equivalent for the present study.

All patients were treated with optimal standard care. Without prior knowledge of the results of cell analysis, antitumor activity was assessed according to criteria that are based on changes in computerized tomo-

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TABLE 1.—Response of ten patients with malignant gliomas to nitrosourea and radiation therapy

Patient population									
				First treatment		Second treatment				
Patient no.	Age	Sex	Location*	Therapy†	Response‡	Therapy†	Response‡	Survival status	Time (wks)	Initial response to nitrosourea‡
1	63	M	L-fr	BCNU	P	Rad	P	Died	17	P
2	50	F	L-fr	BCNU	R	Rad	U	Alive	58+	R
3	69	M	R-fr	Rad+Hu	P	BCNU	P	Died	38	P
4	73	F	L-fr	Rad+Hu	P	CCNU	P	Died	11	P
5	61	F	R-fr	Rad+Hu	R	BCNU	U	Alive	49+	U
6	56	M	L-par	Rad+Hu	R	CCNU+ Proc+VCR	U/P	Died	36	U
7	55	F	R-par	Rad+Hu	R	BCNU	U	Alive	33+	U
8	54	M	R-par	BCNU+FU	U			Alive	13+	U
9	30	F	Medul	BCNU	R			Alive	13+	R
10	22	F	L-fr	BCNU	R			Alive	10+	R

*L = left; R = right; fr = frontal; par = parietal; medul = medullary.
†Rad = radiation therapy; Hu = hydroxyurea; Proc = procarbazine; VCR = vincristine; FU = 5-fluorouracil.
‡P = tumor progressed; R = tumor responded to therapy; U = tumor unchanged.

graphic (CT) and radionuclide (RN) brain scans, and on neurological examination (3).

RESULTS

Tumor cells from Patients 2, 8, 9, and 10 appeared sensitive to BCNU in vitro (fig 1). Patient 2 had subtotal resection of a left frontal glioblastoma and received BCNU at a single dose of 200 mg/m² in the immediate postoperative period. Her brain scan reverted to essentially normal 6 weeks after BCNU administration and she remained clinically well (table 1, fig 2). Patient 8 had subtotal removal of a right parietal glioblastoma and was considered clinically unchanged 6 weeks after a single BCNU dose of 200 mg/m² and a 3-day course of 5-FU (table 1). Patient 9 had subtotal resection of a medullary astroblastoma followed by a single BCNU dose of 180 mg/m². Six weeks later her CT scan demonstrated marked tumor resolution and her neurological deficits improved (table 1). Patient 10 had subtotal removal of a left frontal anaplastic astrocytoma. Six weeks after a single 200 mg/m² dose of BCNU, her CT and RN scans demonstrated marked tumor resolution (table 1).

Cells from six tumor specimens demonstrated resistance in vitro (fig 1). Patient 1 had a biopsy of a left frontal glioblastoma and received BCNU at a single dose of 210 mg/m² in the immediate postoperative period (table 1). His tumor enlarged during the next 6 weeks as shown on CT (fig 3) and RN scans, and his neurological status deteriorated over the treatment period. He died of tumor recurrence 17 weeks after operation despite subsequent radiation therapy. Five patients underwent radiation therapy with concurrent hydroxyurea followed by nitrosourea treatment. Two of these, Patients 3 and 4, progressed after both radiation therapy and subsequent chemotherapy; they died 11 and 38 weeks after operation (table 1). The other three, Patients 5, 6, and 7, received nitrosourea shortly after completing a course of radiation therapy with hydroxyurea that had resulted in dramatic responses (table 1). Patients 5 and 7 received BCNU alone, and Patient 6 received CCNU, procarbazine, and vincristine (PCV); their tumors were considered unchanged 6–9 weeks after nitrosourea administration (table 1).

Tumor cell sensitivity to BCNU in vitro was determined by a greater than 1 log (90%) cell kill for the entire dose range tested and by more than 40% cell kill demonstrated within clinically achievable doses (8.5 μM). The correlation of in vitro tumor cell sensitivity testing and patient response in these ten patients presented is encouraging (table 3).

The untreated CFE did not correlate with either cellular sensitivity or patient response to BCNU (table 2). A maximum cell kill of up to 46% was noted for patients whose cell survival curves implied no response and who appeared to deteriorate clinically while undergoing nitrosourea therapy. This value demonstrates the inherent difficulty in defining a threshold of cell kill that is indicative of significant drug activity.

DISCUSSION

To determine the usefulness of clonogenic cell analysis techniques, potential limitations such as cell selection, a low CFE, differences between treatment in vitro and in situ, and the development of resistance in situ must be considered.

Heterogeneity has been observed in brain tumors by several methods of analysis including histology (4), cell kinetics (5,6), and analyses of clonogenic cells from various portions of the same tumor (2). Furthermore, tumor cells and clones within an individual tumor may be inherently different from one another (7,8). Finally, nonmalignant cells such as fibroblasts, endothelial cells, macrophages, and lymphocytes may be identified in solid tumor (4,9,10) and give rise to colonies in monolayer and soft agar culture systems (10). Therefore, any evaluation of a solid tumor must be made

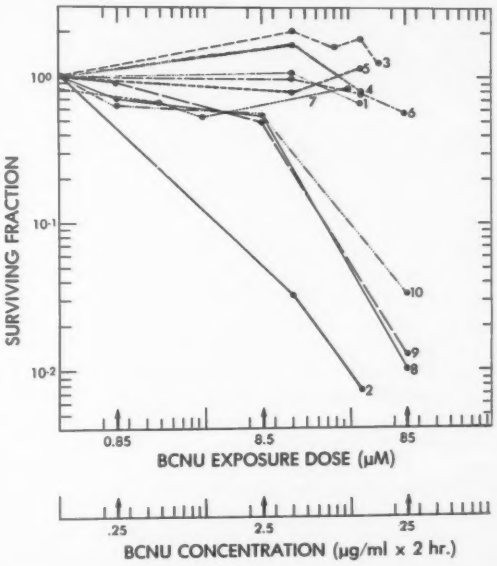

FIGURE 1.—Cell survival curves for tumor cells disaggregated from biopsies of ten human brain tumors and treated in vitro with various concentrations of BCNU for 2 hours at 37°C, then cultured under optimized conditions for 4 weeks. BCNU cellular exposure dose (ED) was calculated according to the equation $ED = A_{(0)}(1 - e^{-k_2 t})$, where a_0 = drug concentration in moles at beginning of treatment; $k_2 = 0.693/64$ min drug half-life; and $t = 120$ min treatment time. A cell exposure dose of 8.5 μM is considered clinically achievable (V. Levin, personal communication. Cells derived from Patients 2, 8, 9, and 10 appeared sensitive to BCNU in vitro; all others appeared resistant.

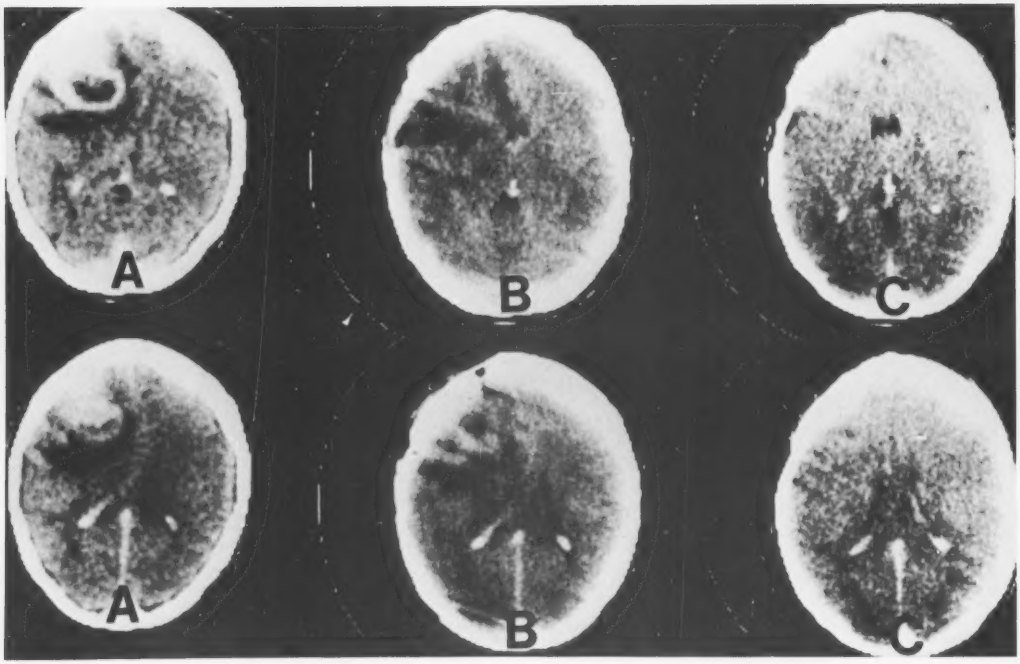

FIGURE 2.—Serial CT brain scans for Patient 2 (A) before surgery, (B) after subtotal resection of a left frontal glioblastoma multiforme, and (C) 6 weeks after a single dose of BCNU and 200 mg/m². The improved appearance in the scans demonstrates a dramatic tumor response to BCNU therapy.

TABLE 2.—Cell survival and patient response to nitrosourea therapy

		Culture				
		Maximum cell kill		Response		
Patient no.	Untreated CFE(%)	(%)	log	Highest dose†	Achievable dose‡	Patient response*
1	0.174	33	0.17	−	−	P
2	0.149	99.3	2.15	+	+	R
3	0.008	0	0	−	−	P
4	0.513	10	0.05	−	−	P
5	0.015	22	0.11	−	−	U
6	0.006	42	0.24	−	−	U
7	0.015	46	0.27	−	+	U
8	0.101	99.0	2.00	+	+	U
9	0.008	98.7	1.89	+	+	R
10	0.047	96.7	1.48	+	+	R

*P = tumor progressed; R = tumor responded; U = tumor unchanged.
†+ = ≥ 90% cell kill at highest dose tested (≥ 34 μM exposure dose).
‡+ = > 40% cell kill at achievable dose (8.5 μM exposure dose).

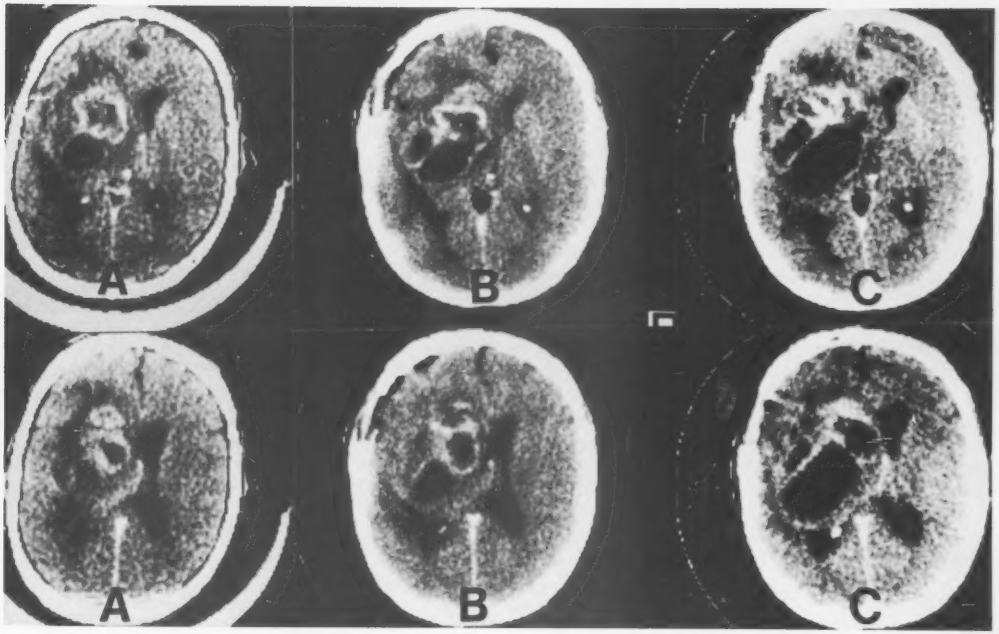

FIGURE 3.—Serial CT brain scans for Patient 1 (A) before surgery, (B) after subtotal resection of a left frontal glioblastoma multiforme, and (C) 6 weeks after a single dose of BCNU at 210 mg/m². The worsening appearance in the CT scans after BCNU corresponds to clinical deterioration and to an increase in the size of the blood-brain barrier defect shown on radionuclide brain scan. These studies demonstrate tumor progression and insensitivity to BCNU therapy.

TABLE 3.—Correlation of tumor response to nitrosourea therapy in culture and in situ

A. Culture response (> 90% cell kill) at highest dose (≥ 34 μM)

Culture	Patient
4 response	3 response
	1 unchanged
6 response	3 progression
	3 unchanged

B. Culture response (> 40% cell kill) at achievable dose (8.5 μM)

Culture	Patient
5 response	3 response
	2 unchanged
5 response	3 progression
	2 unchanged

from multiple specimens from within the tumor mass in an effort to incorporate all representative areas and minimize cell selection as much as possible.

Another problem relates to the fact that only a small percentage of explanted plated tumor cells actually grow in vitro. Using monolayer or soft agar techniques, low CFEs (plating efficiencies) for the clonal growth of human cells from tumor biopsies have been found that ranged from 0.01% to 2% for specimens of bone marrow, ascitic tumor cell suspensions, and solid tumor specimens (2,11–13). The CFE must be increased to accurately define the biology and chemosensitivity of human tissue and tumor specimens. Physical and chemical parameters such as cell size, density, and DNA content may be utilized to isolate and enrich the clonogenic cell population.

The differences in drug delivery to cells in vitro and in situ must also be considered if the chemosensitivity of cultured cells is to be related to tumor response in situ. An understanding of the pharmacokinetics of the chemotherapeutic agents tested is mandatory to test drug concentrations that are potentially achievable at the cellular level in situ and to interpret cell survival analyses (14). In vitro drug testing involves the introduction of agents directly onto the clonogenic cells. In contrast, a drug must penetrate into a solid tumor, pass through the extracellular space, and enter both clonogenic and nonclonogenic cells to affect cell kill (14). Nonclonogenic cells may absorb a drug that in a

purely in vitro assay system would be available to kill clonogenic cells. Variations in drug delivery within an individual tumor may occur consequent to variations in vascularity, cellular incorporation, and metabolism. Drug delivery might be complicated by the fact that after several drug courses tumor proliferation can take place primarily at sites where cytotoxic concentrations of drug had not been achieved. Nevertheless, if drug resistance is noted in vitro, tumor resistance in situ might be anticipated.

Exposure of the tumor to a proteolytic enzyme or other agents may alter the cytotoxic activity of subsequently administered chemotherapeutic agents due to either changes in drug penetration, drug activity, or cellular repair processes.

The last consideration is theoretic. Tumor resistance may develop after two or more drug cycles, which would not be anticipated from the initial selection of an antitumor agent. If a biopsy were possible at that time, it would be possible to select new, potentially sensitive agents; however, a repeat biopsy is frequently not feasible. The simultaneous administration of several agents that have each shown potential activity against a tumor might decrease the rate of development of drug resistance, compared to treatment with each agent individually. Investigations into the fundamental biology of tumor resistance are necessary to understand and combat this problem.

FUTURE PROSPECTS

Despite the potential limitations described above, if a correlation can be demonstrated between in vitro drug sensitivity testing and in vivo patient response, clinical application of the cellular assay system may be possible.

Salmon et al (1) have shown a strong correlation between the results of in vitro testing and patient response for multiple myeloma and ascitic ovarian carcinoma. Clinical trials utilizing chemotherapeutic agents selected on the basis of cell sensitivity analyses are already underway at their institution.

As the limitations of methods are better understood, human tumor cloning should be used to investigate and evaluate the clonogenic tumor cell population, its heterogeneity, kinetics, and drug sensitivity. Furthermore, an evaluation of tumor resistance may be possible.

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Proliferation of Glioma Cells in Serum-Free Defined Medium[1]

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SUMMARY

The requirement for a serum supplement to basal culture medium to support the growth and viability of cells in vitro has been a major hindrance to reducing and thereby controlling experimental variables. The complexity and undefined nature of serum as well as the variability of serum lots make it difficult to reproduce data and to interpret results of experiments. This paper presents preliminary results obtained with C6 glioma cell cultures in which serum was replaced by growth factors and a hormone. A brief review of the use of serum-free defined medium for the culture of tumor cells and the potential uses of defined media that are cell-type specific are also presented.

[Cancer Treat Rep 65 (Suppl 2):67–70, 1981]

Although the regulation of tumor cell growth has been extensively studied, the relationship between proliferation of cells and (*a*) hormones, (*b*) growth factors, and (*c*) substrata is poorly understood. In vitro studies are complicated by the requirement for a serum supplement to basal medium to support cell growth and viability. Generally, tumor cells will not grow in unsupplemented basal medium. Theoretically, in vitro systems offer the advantage of greater control of experimental variables; however, because it is complex and undefined, serum added to culture hinders control. The variability of serum lots may be in part responsible for the irreproducibility of some experiments. In addition, serum contains a variety of known hormones, growth factors, and differentiation factors that can make the study of their actions difficult if they cannot be depleted readily from serum. Furthermore, the effect of exogenous agents may be modulated by or absolutely require a critical concentration of one or more undefined serum constituents. Thus the study of the regulation of tumor cell growth in vitro can be conducted best with serum-free medium.

MATERIALS AND METHODS

Materials

Materials were obtained from these sources: DME (H-21) medium, F-12 (H-17) medium, and horse serum from Gibco, Grand Island, NY; fetal calf serum from Reheis, Phoenix, AZ; trypsin (1–300) from ICN Pharmaceuticals, Cleveland, OH; EDTA, HEPES, soybean trypsin inhibitor, poly-D-lysine-HBr (B-1; MW 150,000), crystalline bovine insulin, human transferrin, progesterone, and putrescine dihydrochloride from Sigma, St. Louis, MO; sodium selenite, Johnson Matthey Chemicals, from their distributor United Mineral and Chemical, New York, NY; FGF and EGF from Collaborative Research, Waltham, MA; tissue culture plasticware from Falcon Plastics, Oxnard, CA.

Cell Culture

The CHP-134 human neuroblastoma cell line was obtained from R. Seeger (Department of Pediatrics, University of California, Los Angeles) and the C62B rat glioma cell line from J. de Vellis (Department of Anatomy, University of California, Los Angeles). Both cell lines were maintained as stock cultures in a 1:1 mixture of Ham's F12 medium and Dulbecco-Vogt's modification of Eagle's medium (DME) supplemented with 20% fetal calf serum (CHP-134) or 5% horse serum/2.5% fetal calf serum (C62B). Both stock media contained 1.2 g/l of $NaHCO_3$; 15 mM HEPES; and 40 mg/l penicillin, 8 mg/l ampicillin, and 90 mg/l streptomycin. Triple-distilled water was used to prepare all media. Cultures were grown in a humidified atmosphere of 5% CO_2–95% air at 37°C and were subcultured just before confluence every 4–6 days with a 0.1% trypsin solution containing 0.9 mM EDTA in Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS; pH 7.3).

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Experimental Media

Basal medium had the same constituents as stock culture medium (see above) with the deletion of sera. Experimental media were always made up fresh immediately before use because their stabilities under storage are presently unknown. Fibronectin was affinity-purified from human plasma (1,2); it was always added before the cell inoculum to dishes containing medium, which were immediately tilted to insure even distribution.

Trypsin Detachment of Cells

A trypsin solution (see above) was used to detach cells from the substratum after a PBS wash. Excess trypsin solution was decanted and cells were held at ambient temperature in the residual trypsin solution until a vigorous tap dislodged them. To inactivate the trypsin, serum-containing medium was added for stock cultures or a 0.1% solution of soybean trypsin inhibitor in PBS for serum-free cultures. Cells were centrifuged to obtain a cell pellet, which was resuspended in the appropriate medium and inoculated into new dishes.

RESULTS

A partially defined serum-free medium for growth of C6 cells has previously been described (3). Cells were inoculated into serum-containing medium; after 24 hours they were washed three times and switched to a defined medium consisting of basal medium (1:1 F12/DME) supplemented with 5 μg/ml transferrin, 2 μg/ml insulin, 20 ng/ml fibroblast growth factor, and 10 μg/ml of an extract of rat submaxillary gland. In this mixture C6 cells grew at a rate comparable to that in the optimal serum-containing medium (6%). The submaxillary factor(s) could not be replaced by purified nerve growth factor or epidermal growth factor.

This procedure required a serum preincubation and use of the undefined submaxillary gland extract. Table 1 lists the results of an experiment in which neither of these are present. In defined medium C the growth rate is 70% of that in serum-containing medium B. Medium D is the same as medium C with the addition of 50 ng/ml of epidermal growth factor (EGF), which inhibits the growth of C6 cells. Lower concentrations of EGF have no effect, unlike the response of a normal human glial cell line, which had an increased incorporation of thymidine when treated with 1 ng/ml EGF and 1 mg/ml human serum albumin in Ham's F12 medium lacking serum (4).

DISCUSSION

Various investigators have attempted to grow cells in basal medium without serum (5–7). This procedure is an adaptive one, however, in which cells that have lesser exogenous growth requirements are selected for. In some cell lines it is possible to lower serum requirements, but further reduction results in no cell growth and the frequent loss of differentiated functions. Moreover, it is often impossible to clone cells under these conditions. Another problem is that each cell line must be adapted to serum-free medium.

An alternative approach is to replace serum with complexes of hormones and growth factors and to provide the appropriate substratum. This nonadaptive technique has been used successfully to grow a number of tumor cell lines (3,8–10). Table 2 lists a represen-

TABLE 1.—Growth of C6 rat glioma cells in various media

Medium	Cells/35mm dish × 10^{-5}	Doubling time (hr)
A: basal	0.33±0.01	—
B: basal + 10% FCS	3.43±0.12	26
C: basal + insulin, 5 μg/ml transferrin, 5 μg/ml FGF, 10 ng/ml fibronectin, 5 μg/ml	1.62±0.02	37
D: basal + insulin transferrin FGF fibronectin EGF, 50 ng/ml	1.14±0.03	48

Inoculum was 30,000 cells/35-mm dish; cells were counted on Day 4 with a Coulter counter. Media C and D were added to polylysine precoated dishes (13) and fibronectin was added just before the inoculum. Basal medium constituents are described in the Materials and Methods section.

TABLE 2.—Growth requirements of tumor cells in serum-free medium

GH_3 pituitary tumor (Ref 3)	HeLa cervical carcinoma (Ref 3)	M2R melanoma (Ref 3)	B 104 neuroblastoma (Ref 10)
Insulin, 5 μg/ml	Insulin, 5 μg/ml	Insulin, 5 μg/ml	Insulin, 5 μg/ml
Transferrin, 5 μg/ml	Transferrin, 5 μg/ml	Transferrin, 5 μg/ml	Transferrin, 100 μg/ml
T_3, 300 nM	Hydrocortisone, 30 nM	Progesterone, 1 nM	Progesterone, 20 nM
TRH, 1 ng/ml	EGF, 1 ng/ml	LRF, 10 ng/ml	Putrescine, 100 μM
PTH, 0.5 mg/ml	FGF, 0.1 μg/ml	FSH, 40 μg/ml	Selenium, 30 nM
FGF, 1 ng/ml	Trace element mixture	NGF, 10 ng/ml	(N2 medium)
Somatomedin C, 1 ng/ml		Selenium, 0.1 μM	

The above supplements were added to basal medium. See references for details of culture procedure.

tative sample of tumor cells that can be grown in such supplemented media, all of which require both insulin and transferrin, as most other cell lines cultured in defined medium do. However, it is apparent that the requirements differ for each cell type.

The GH_3, HeLa, and M2R cell lines do not require any modification of the surface of the culture dish. However, the B104 cell line will not proliferate if plated directly into serum-free supplemented medium; it will proliferate if inoculated into serum-containing medium and switched to supplemented medium after 18 hr (8), or if the substrate is precoated with poly-D-lysine and 5 μg/ml fibronectin is added to the supplemented medium (10). Differentiated properties of B104 cells are retained in this medium (8) and it will support the proliferation of several subclones of C1300 mouse neuroblastoma (11) and LA-N-1 human neuroblastoma (9). Figure 1 shows the effect of B104 medium on CHP-134 human neuroblastoma cells (12); it supports their proliferation but does not permit continuous growth of the C62B rat glioma or the Balb/c 3T3 mouse fibroblast cell line (9).

Although glial tumor cells are of primary interest to investigators of brain tumors, there have been no reports of a rigorously defined serum-free medium for their optimal growth. Donta has adapted the C6 rat glioma cell line to grow in Charity Waymouth serumless medium without loss of production of the brain-specific protein S-100 (7). However, the growth rate was only 30% of that in serum-containing medium. These cells have also been adapted to grow serum-free in Ham's F10 medium (14). More recently, a partially defined serum-free medium for growth of C6 cells has been described (3).

While the growth rate of C6 cells in the defined medium described here is somewhat lower than in serum-containing medium, these culture conditions may be suitable for many experimental purposes. Serial subcultures have not yet been attempted in the supplemented medium, nor have any differentiated properties been evaluated. It appears that additional factors may be required to obtain a growth rate in defined medium that is comparable to serum-containing medium. Currently we are attempting to define the requirements for serum-free growth of a human glioma cell line in which only well-defined factors will be utilized. We anticipate that in analogy to the neuroblastoma medium (8), this defined medium will be similar or identical to one that supports proliferation of the C6 rat glioma.

CONCLUSION

It appears that nonadaptive serum-free culture conditions can be identified for specific tumor cell types. The uses of such rigorously defined media are then quite diverse. They include studies of growth regulation, cell-substratum and cell-cell interactions, identification and isolation of tumor-produced molecules, mechanism(s) of action of growth-stimulatory and -inhibitory molecules, and inducers and regulators of differentiation. Knowledge of the specific growth re-

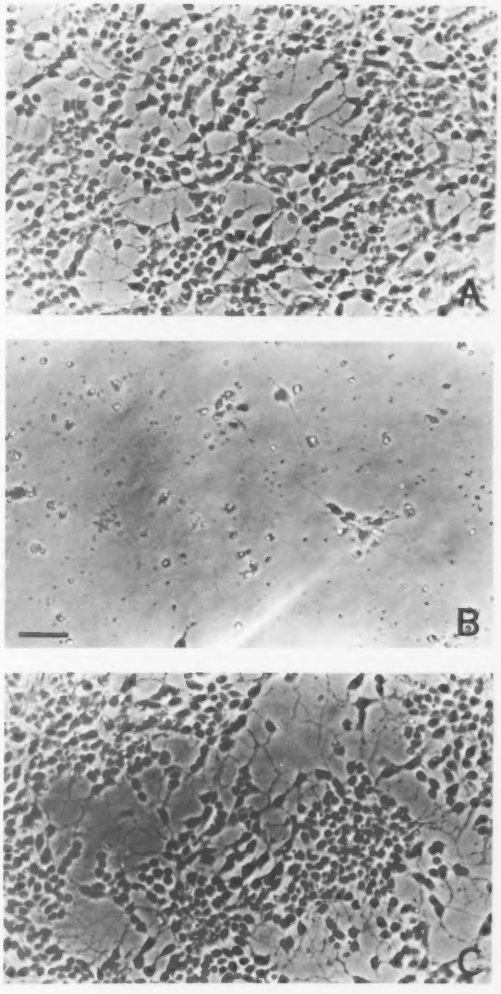

FIGURE 1.—Phase contrast micrographs of CHP-134 human neuroblastoma cells in various media on Day 5. Inoculum was 200,000 cells/35-mm dish. Panel A: basal medium with 30% fetal calf serum. Panel B: basal medium alone. Panel C: N2 medium with 5 μg/ml fibronectin added and a polylysine (13) precoated dish. Basal medium constituents are given in the Materials and Methods section and N2 medium supplements are listed in table 2. Scale bar, 50 μm.

quirements of tumor cells could conceivably be exploited to design new forms of therapy. Finally, cell-type specific media that are most easily formulated using tumor cell lines may be used to select for their normal counterparts in primary cultures containing mixed populations of cell types (15,16). There is then the potential for comparing normal and tumor cells of the same type with respect to their growth properties or other parameters.

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Heterogeneous In Vitro Sensitivities of Metastatic B16 Melanoma Sublines and Clones to Retinoic Acid or BCNU[1]

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SUMMARY

Murine B16 melanoma sublines have been cloned or selected in vivo for preference of bloodborne metastatic colonization of lung, ovary, or brain. These sublines show differing metastatic properties and cell surface alterations that correlate with a preference for metastatic colonization sites. When the sensitivities of these selected sublines to certain drugs (β-all-trans retinoic acid or 1,3-bis (2-chlorethyl)-l-nitrosourea (BCNU) were examined, the in vivo-selected sublines were more resistant to growth inhibition in vitro by cytostatic (retinoic acid) or cytotoxic (BCNU) drugs than was the parental B16 line.

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The development of distant metastases involves several discrete and highly selective steps (1–3), but not all malignant cells from a primary tumor survive each step. It has been suggested that successful therapy may depend on still-to-be developed chemotherapeutic agents that eliminate the few potentially fatal metastatic cell subpopulations that exist within a malignant tumor (4).

In order to study the metastatic cells within a tumor cell population, malignant cell sublines with different metastatic potentials have been obtained by sequentially selecting cells for enhanced bloodborne implantation, survival, and growth (3,5). Fidler (6) used a murine B16 melanoma line with moderate in vivo metastatic potential to select variant sublines with high lung colonization potential. After one selection for lung colonization, a line was obtained (B16-F1) that after nine additional in vivo selections yielded a line (B16-F10) more metastatic than either parental B16 or B16-F1 melanoma lines (6,7). Using similar selection approaches, Brunson and Nicolson obtained variant B16 sublines with increased abilities to colonize the brain (B16-B10N, B16-B10B) (8,9) or ovaries (B16-010) (10). In addition, some of the in vivo-selected sublines (B16-F1 and B16-F10) have been further selected in vitro to obtain the lymphocyte-resistant (Lr) sublines B16-F1$^{Lr\text{-}6}$ and B16-F10$^{Lr\text{-}6}$ (11).

Heterogeneity in the phenotypic properties of cells in an unselected parental B16 melanoma population has been demonstrated by in vitro experiments in which clones of widely differing metastatic potential were obtained (12). Fluctuation assays demonstrated that the highly metastatic clones were present in the original tumor cell population and were not generated by the cloning procedures (12). It has been found that other kinds of tumors possess rare variant subpopulations that have high metastatic potential (5,13–15).

In this study, we examined the effects of cytostatic retinoids (16,17) and cytotoxic nitrosoureas on unselected and selected B16 melanoma sublines and their clonal derivatives (18). Retinoids are currently being used in clinical trials in patients harboring metastatic melanoma (19) and have been found to inhibit in vitro growth of untransformed, transformed, and tumor cell lines (20). The nitrosoureas are active against a wide variety of solid tumors, including malignant melanoma (21), and against several types of metastatic tumors (22). Here we examine the ability of

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retinoic acid or BCNU to inhibit the proliferation of parental B16 melanoma, some of its clones, and several in vivo-selected variant sublines with variable metastatic potentials.

MATERIALS AND METHODS

Cells and Culture Techniques

Cells used in this study were derived from a single cell line established by Dr. I. J. Fidler (NCI-Frederick Cancer Research Center, Frederick, MD) from a transplantable malignant murine B16 melanoma of spontaneous origin (Jackson Laboratories, Bar Harbor, ME). Metastatic lines B16-F1, B16-F10, and their lymphocyte-resistant sublines were obtained from Dr. Fidler, as well as some of the clones derived from the parent B16 melanoma line, B-16-F1$^{Lr\text{-}6}$ and B16-F10$^{Lr\text{-}6}$. The metastatic cell sublines B16-B10N, B16-B10B, and B-16-010 were developed in our laboratory from the line B16-F1.

All experiments were carried out with early passage cells (<10) cultured in 10-cm diameter tissue culture dishes (Falcon Plastics, Oxnard, CA) with Dulbecco-modified Eagle's minimal essential medium containing 10% heat inactivated fetal bovine serum (Flow Laboratories, Inglewood, CA) and nonessential amino acids. Cultures were incubated at 37° in a humidified atmosphere of 5% CO_2-95% air. For passage and enumeration, cells were detached with 2 mM EDTA-phosphate-buffered NaCl solution, pH 7.2, and counted on an electronic particle counter (Electrozone/Celloscope, Model 112ct, Particle Data, Inc., Elmhurst, IL) or with a hemacytometer. Doubling times of various cell lines were determined by following their growth from an inoculum of 5×10^4/dish for 5 days with media changes on alternate days (18). Cell viabilities were determined by phase contrast and from the proportion of cells that excluded trypan blue.

Assay for Growth Inhibition

β-All-trans retinoic acid (a gift of Hoffmann-La Roche, Inc., Nutley, NJ) was dissolved in ethyl alcohol to obtain a series of decreasing concentrations of 1×10^{-2}M to 1×10^{-6}M, which were stored under nitrogen at −20°C for up to one week. Immediately before each experiment, retinoic acid solutions were diluted 1:1000 into culture medium to give a final alcohol concentration of 0.1%. Control cultures received media containing 0.1% alcohol, and all procedures were carried out in reduced light (18).

In some experiments cells were plated at a density of $0.5\text{–}5 \times 10^4$/dish in 10 ml of media in the presence or absence of drugs at various concentrations on Day 0. Media were changed on Day 3, and the cells were detached and counted on Day 5. The percent growth inhibition was calculated from the equation $100 - (D/C) \times 100$, where D and C are the number of cells in drug and control cultures respectively (19). For BCNU inhibition of B16 melanoma cell growth, 5×10^3 cells were plated on Day 0, and various concentrations of BCNU were added on Day 2. Cell numbers and viabilities were determined on Day 6, and the percent difference from the control calculated.

RESULTS

B16 melanoma sublines used in these experiments had doubling times of 14–16 hours, except for B16-B10N (17–18 hours) and B16-010 (17–19 hours); the plating efficiencies of the lines were always 70%–80%. Melanin produced by the various lines and sublines varied widely in amount and showed no obvious relationship between melanogenesis and malignancy (23).

Subculturing B16 melanoma sublines in the presence of 10^{-5}M retinoic acid did not modify their plating efficiencies or viabilities. Although some B16 sublines were less affected by retinoic acid, by Day 5 all cell lines were growth-inhibited. Parental B16 and B16-F1 lines, which are especially sensitive to retinoic acid, were growth inhibited by ≃ 90%, compared to controls. Decreasing concentrations of retinoic acid were then used; growth inhibition caused by retinoic acid was concentration dependent (18), although some of the B16 sublines were not very sensitive to the drug. At the lowest retinoic acid concentration tested (10^{-9}M), parental B16 and B16-F1 lines were growth-inhibited nearly 40%, but sublines B16-010, B16-F10 and B16-B10N were not significantly inhibited.

Because the dose-response relationships were almost linear for the B16 cell lines (18,23), the concentration of retinoic acid required for 50% growth inhibition (I_{50}) of the various lines could be compared (table 1). Line B16-F1 had an I_{50} value similar to parental B16, in marked contrast to the I_{50} for sublines B16-B10N, B16-F10$^{Lr\text{-}6}$, B16-010, and B16-F10. The greatest difference in retinoic acid sensitivity was ≃ 1000-fold (B16-F10 vs B16 parental or B16-F1). That the retinoic acid sensitivities of the various B16 sublines are a stable cell characteristic was demonstrated by long-term continuous weekly subculture (3 months) in the presence of 1×10^{-5}M drug. After this period the I_{50} for retinoic acid did not vary by more than 10% from that of cells grown in the absence of drug (18).

Differences in the sensitivities of the various B16 cell lines to BCNU were also found. Every in vivo-selected subline tested was less susceptible than line B16-Fl to the cytotoxic effects of BCNU, which were 25–50 times more resistant, based on I_{50} in tissue culture (table 1).

We examined the effects of retinoic acid on the proliferation of individual parental B16 clones chosen

regardless of their implantation properties (table 2). Although all of the B16 parental clones were more sensitive than their various sublines to retinoic acid-induced growth inhibition, the parental lines' I_{50} varied between 0.06 μM to >10 μM, there was no obvious relationship between drug sensitivity and implantation potential. In addition, we tested the effects of retinoic acid on the proliferation of cells in sparsely plated cultures of B16-Fl and found a similar pattern in the growth inhibition of cells from individual, randomly selected colonies and in cells from the whole cell population (table 3). Although most cell colonies consisted of retinoic acid-sensitive cells, the presence of a few less sentitive cell subpopulations confirmed the existence of drug-resistant variants among the original cell population (18).

DISCUSSION

Chemotherapeutic agents are only partially successful in treating disseminated metastatic disease (22). Their effectiveness may be improved if their ability to eliminate highly metastatic cells in the parental tumor cell population can be defined. Thus we have

TABLE 1.—Inhibition of in vivo-selected B16 melanoma sublines by retinoic acid or BCNU

B16 subline	Metastatic potential	Organ colonization preference	Approximate concentration required for 50% growth inhibition by retinoic acid (μM)	Approximate concentration required for 50% growth inhibition by BCNU (μg/ml)
F1	Moderate	Lung > lymph node > brain > ovary, liver, thoracic	0.01	0.1
F10	High	Lung	> 10	2.5
$F10^{Lr\text{-}6}$	Low	Lung	1.0	ND*
B10N	Moderate	Brain > > lung	0.07	2.5
B10B	Moderate	Brain > lung	ND*	2.5
O10	High	Ovary > lung	10	5.0

*ND = not determined.

TABLE 2.—Inhibition of B16 melanoma clones by retinoic acid

Parental clone	Metastatic potential	Organ colonization preference	Percent growth inhibition by 10^{-5}M retinoic acid	Approximate concentration for 50% growth inhibition (μM)
Parental line	Moderate	Lung > lymph node > ovary > liver > brain, kidney	88 ± 4	0.01
Clone 12	Low	Lung	62 ± 3	0.3
Clone 15	Low	Lung > lymph node	40 ± 2	> 10.0
Clone 9	High	Lung > lymph node > brain	51 ± 3	1.0
Clone 14	High	Lung > > ovary	85 ± 3	0.06

TABLE 3.—Inhibition of population and colony growth of B16-Fl melanoma cultures by retinoic acid

Day	Percent inhibition of growth in 10^{-5} M drug*	
	Whole population†	Individual colonies‡
1	0	0
2	0	0
3	30.4	28.2
4	64.0	61.5
5	86.2	81.9

*Cells from the same culture were plated at 10^2, 10^3, or 10^4 per 6-cm dish in 5 ml growth medium in the absence or presence of 10^{-5} M retinoic acid.
†At 24-hour intervals thereafter, duplicate cultures were removed and the total number of cells per dish determined. The values represent the ratios of the means ± drug (SEMs ranged from 3.5%–9% of raw data).
‡At 24-hour intervals thereafter, duplicate cultures at different cell densities were removed, fixed, and stained with Diff-Quik (Harleco, Gibbstown, NJ) and the number of cells per colony determined. The values represent the ratios of the means ± drug (SEMs ranged from 13%–19% of raw data) of cells per colony in 50 individual colonies in each duplicate (18).

begun an investigation into the drug sensitivities of various parental tumor lines, their clones, and in vivo-selected highly metastatic derivatives. Our first studies with retinoids and B16 cell lines (18,23) indicated that considerable variability existed in the dose response of each subline or parental clone. Although tumor cell clones are known to differ significantly in their sensitivities to cytostatic drugs (24), only a few studies (25) have been performed on cell lines or clones with disparate metastatic properties.

We found no obvious relationship between the sensitivity to retinoic acid of a B16 melanoma cell line or clone and its metastatic potential in vivo. Some cell clones obtained from parental B16 melanoma were almost as sensitive to retinoic acid as the parental line, although they differed significantly in implantation properties. However, we observed all the in vivo-selected B16 sublines to be more resistant to retinoic acid or BCNU than the parental B16 or line B16-F1. This finding suggests that in vivo selection for enhanced metastatic colonization could yield cell variants with altered drug resistance. In our previous study, an analysis of growth inhibition of at least 100 individual B16-Fl colonies cultured in the presence of 10^{-5}M retinoic acid confirmed that most cell colonies are inhibited by the drug to a similar extent; however, there were a minor number of less drug-sensitive cell colonies (18).

Our studies demonstrate that the in vitro drug sensitivities of uncloned, unselected tumor cell lines may not prove useful in predicting drug sensitivities of rare metastatic variant sublines or clones that exist in the mixed tumor cell population. These highly metastatic cell subpopulations can lead to disseminated tumor progression after surgical removal of the primary tumor.

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Review of Factors Influencing the Response of Experimental Brain Tumors to Therapy[1]

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SUMMARY

This review summarizes the problems and pitfalls encountered when increased life span studies are performed with brain tumor model systems. The biological factors reviewed include tumor size and growth rate, the immune response, cell kinetics, and cell recovery processes. The analytical factors reviewed include data collection, data presentation, covariate analysis, and statistical analysis. Examples of how these factors can influence the ultimate interpretation of results from brain tumor therapy trials are presented.

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For the past several years we have studied the effects on brain tumor cells of combinations of BCNU and radiation in an attempt to develop a more rational basis for optimizing the use of these agents in the treatment of human brain tumors (1,2). Initially these experiments concentrated on elucidation of the interactions that occurred when cultured cells were treated. Low doses of BCNU enhanced the cell-killing effect of radiation, but the magnitude of this enhancement appeared to be time-dependent, and was maximum when BCNU was administered 16 hours before irradiation (1). Isobologram analyses of these data indicated that the BCNU-radiation interaction was additive and that BCNU produced its cell kill by the same mechanism as ionizing radiation (3). However, the exact mechanism responsible for this enhanced cell kill remained unknown.

Although most of these in vitro studies were performed on 9L rat brain tumor cells, the results were in general agreement with those obtained using other cell lines or tissue (4,5). 9L cells did not recover from BCNU-induced potentially lethal damage (PLD) or sublethal damage (SLD) when treated either in vitro or in situ (6,7). However, these same 9L cells showed considerable recovery from radiation-induced PLD or SLD (8,9). Nevertheless, concentrations of BCNU that interacted with radiation to enhance cell kill (1) did not appear to significantly interfere with recovery from radiation-induced PLD or SLD (K. T. Wheeler, unpublished data). Because interference with cellular recovery processes did not seem to be responsible for the time-dependent BCNU-radiation interaction, this interaction might be an artifact associated with the use of cultured cells in these experiments. Consequently we decided to perform experiments in male Fischer 344 rats with 9L cells implanted intracerebrally (ic) according to the methods of Barker et al (10), and use increase in life span as the analytical endpoint to determine if low doses of BCNU would show a time-dependent enhancement of the response of in situ irradiated brain tumors similar to that obtained with 9L cells in vitro.

It was immediately obvious that the design of experimental brain tumor therapy trials was much more complicated than similar trials using solid tumors at other sites. Brain tumors grow within the cranial cavity and kill the animal by compressing vital central nervous system structures; therefore, the time of death may depend on the location of the tumor. Because theoretically tumor size may be the primary determinant of death, anything that contributes to an increase in intracranial mass (edema, hemorrhage in the tumor, host cell infiltration, etc.) may influence the time of death. Also, increases in the mass within the cranial cavity can occur because, (*a*) dead cell removal is apparently slower in the brain than in other parts of the body (11), (*b*) clonogenically sterile cells may continue to

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metabolize and become giant cells, or (*c*) cells may go through three or more divisions before they die, lyse, and are removed (12).

Furthermore, the rate at which a tumor grows in the brain could determine the size a tumor must be to kill an animal; a faster-growing tumor may kill an animal at a smaller size than a slower-growing tumor because the brain has more time to adapt to the increase in mass. The exact manner in which the animal dies from a brain tumor can also influence the time of death (10). Animals often become lethargic, comatose, and can neither eat nor drink. If death is not immediate, dehydration can reduce the effects of edema and allow the tumor to grow to a larger size before it kills the animal, thus biasing the life span data. Other important factors that must be considered include the following: (*a*) brain tumors are invasive and can seed throughout the central nervous system; (*b*) the brain may be a relatively immunologically privileged site; (*c*) the blood-brain barrier can be intact in some portions of the tumor and defective in others (13). Finally, this entire scenario is played inside a closed box in which the investigator can neither see nor measure the dynamics of any of these factors during the life of the animal. Only autopsy results are observable. Consequently, treating tumors of a known uniform size is at best problematic. Because every agent in a therapy scheme can affect any or all of these factors, the interpretation of data based on increases in life span and their extrapolation in predicting the response of human tumors to therapy becomes exceedingly complex.

INFLUENCE OF BIOLOGICAL FACTORS ON LIFE SPAN STUDIES

Tumor Size and Growth Rate

In order to examine the influence of tumor size at treatment and tumor growth rate on the apparent response of brain tumors to therapy, we isolated two sublines of the 9L tumor, designated 9L/Ro and 9L/SF (9). When 5×10^3 9L/Ro cells were implanted ic in male Fisher 344 rats, the median day of death for untreated rats was 16–18 days; for 9L/SF, the corresponding median day of death was 23–25 days. When rats bearing these tumors were treated with fractionated schedules of BCNU, x-rays, or combinations of these agents, the observed increase in life span and the number of long-term survivors depended only on the size of the tumor at treatment (table 1). Differences in the tumor growth rate (9L/Ro tumor weight doubling time (TWDT) ≈ 39 hours; 9L/SF TWDT ≈ 48 hours) did not appear to determine life span or the number of long-term survivors, even though these growth rates caused differences in the size of the tumors (9L/Ro = 100–150 mg, 9L/SF = 200–300 mg) that killed the rats (9). Consequently, all comparisons of various therapies must be made with brain tumors treated at the same size. Although the median day of death of untreated tumor-bearing rats is a good indicator that tumors treated on a given day were of a given size, it is safer to include all comparison groups in the same experiment.

Immune Response

The influence of the immune system on brain tumor response is somewhat elusive. Because the brain is a relatively privileged sanctuary, the potential role of the immune system on brain tumor therapy results tends to be ignored. The TD_{50}* for 9L/Ro cells implanted in the brain was less than 10 cells and perhaps as low as one clonogenic cell (table 2). The TD_{50} for 9L/SF cells was slightly greater than 10 cells (table 2). However,

*TD_{50} is the number of implanted cells required to produce tumors in 50% of the animals.

TABLE 1.—Comparison of response of ic 9L/Ro and 9L/SF tumors to fractionated treatment with drugs and radiation

	Initial day* of treatment		Increased life span (%)		Fraction tumor-free	
Treatment	9L/Ro	9L/SF	9L/Ro	9L/SF	9LRo	9L/SF
None	—	—	—	—	0/10	0/10
BCNU	10	14	40	36	0/10	0/10
x-Ray	10	14	26	12	0/9	0/10
BCNU + x-ray	10	14	NA†	NA	6/10	6/10
BCNU	12	17	26	34	0/10	0/10
x-Ray	12	17	20	0	0/10	0/10
BCNU + x-ray	12	17	NA	NA	5/9	9/10
BCNU	14	20	14	18	0/9	0/10
x-Ray	14	20	3	0	0/10	0/10
BCNU + x-ray	14	20	71	65	2/10	1/10

*9L/Ro and 9L/SF tumors are identical in size on the days compared (9).
†NA = not applicable.

TABLE 2.—Percentage of immunized and nonimmunized rats that died from 9L brain tumors at various times after implantation

Tumor subline	Cells implanted	Days postimplantation 20	30	40	50	60	70
9L/SF	5×10^3	14	71	100	100	100	100
(nonimmunized)	10^3	0	57	100	100	100	100
	5×10^2	0	43	43	100	100	100
	1×10^2	0	0	14	86	100	100
	50	0	0	0	14	43	71
	10	0	0	0	14	43	43
9L/SF	10^5	57	100	100	100	100	100
(immunized)	10^4	0	14	57	57	57	57
	10^3	0	29	29	29	29	29
9L/Ro	5×10^3	100	100	100	100	100	100
(nonimmunized)	10^3	29	100	100	100	100	100
	5×10^2	0	71	100	100	100	100
	10^2	0	100	100	100	100	100
	50	0	57	57	86	100	100
	10	0	0	0	17	83	83
9L/Ro	10^5	100	100	100	100	100	100
(immunized)	10^4	86	100	100	100	100	100
	10^3	0	0	14	14	29	29

these low TD_{50} values did not necessarily mean that the tumor was nonimmunogenic. The TD_{50} for 9L/Ro cells implanted sc in the inguinal region of the leg was $\approx 3 \times 10^4$ cells, suggesting that 9L/Ro tumors might be highly immunogenic (1). To test this hypothesis rats were preimmunized with an ip injection of 1×10^7 heavily irradiated (4000 rads) 9L/Ro or 9L/SF cells and then challenged 14 days later with various concentrations of unirradiated cells. The intracerebral TD_{50} increased to $> 10^3$ cells for both 9L/SF and 9L/Ro cells (table 2). The TD_{50} for 9L/Ro sc tumors in similarly preimmunized rats or rats preimmunized by excising sc tumors or by curing the tumors with heat was $> 2 \times 10^7$ cells. These results demonstrate that both 9L tumor lines are moderate to highly immunogenic and that an immune response against ic immunogenic tumors can be generated by an animal.

The immune system certainly contributes to the effective cell-kill achieved in most 9L tumor therapy schemes because schedules that produce a 3 log cell kill in 10–50 mg tumors ($1–5 \times 10^7$ cells) occasionally result in some rats being "cured" (7). In addition, the clonogenic efficiency of 9L cells from ic tumors increases from about the 30% observed in normal rats to about 60% in immunosuppressed rats, a value similar to that obtained for cultured 9L cells. Therefore, a low TD_{50} in nonimmunized rats can easily lull the experimentalist into a false sense of security. Although the immunogenic character of the virally-induced brain tumor models is recognized by most investigators, little is known about the immunogenic characteristics of most of the other brain tumor models. In general, the effects of this parameter on the interpretation of life span data tend to be ignored. To do so inserts the assumption that the immunogenic character of the experimental model must be exactly like that of human brain tumors if the results are to be extrapolated to the design of clinical trials. Clearly, considerable information on the immune system and its effects on increased life span data is required to correctly interpret the results of mortality studies in most brain tumor models.

Cell Recovery Processes

Cell recovery processes are another factor that may influence the response of a brain tumor to therapy. We have recently demonstrated that cell survival measured by an in vivo to in vitro colony-forming assay immediately after irradiation was not a determinant of the increase in life span in 9L brain tumors (9). Rather, the cell survival measured 24 hours after irradiation, when recovery from x-ray-induced PLD was complete, determined the increase in life span (9). On the other hand, because 9L brain tumor cells exhibit no recovery from BCNU-induced PLD, measurements of cell survival 30 minutes after treatment correlated with the increase in life span after BCNU treatment (7). Although the absence of recovery from nitrosourea-induced PLD has been reported for other cells (15,16), it is by no means a universal phenomenon. Recovery has been demonstrated in both EMT-6 and KHT tumors (17–19; D. Siemann and T. Mulcahy, personal communication). Since such phenomena can easily produce discrepancies that complicate the extrapolation of experimental data to the clinical situation, their role and its universality must be considered when interpreting results from increased life span studies.

Cell Kinetics

Although the number of cells killed by a treatment is not the only factor that can influence tumor response, for most treatments it is a major determinant of response. Clearly, however, changes in the life span of tumor-bearing animals (or patients) can also result from changes in the growth fraction or in the cell cycle time. Apparent changes in growth fraction have been found for 9L brain tumors after treatment with single doses of BCNU (20). For most agents, treatment increases the growth fraction, a clearly undesirable situation. However, both BCNU and x-rays do not appear to substantially alter the cell cycle time of 9L cells although the cell doubling time is altered (20,21). If changes in life span produced by an experimental therapy reflect only changes in cell kinetic parameters, then use of this therapy by itself in the clinic can be expected to prolong life but not alter the ultimate outcome, ie, it would not produce cures. It is therefore important to know that increases in life span, and not just cell kinetic changes, are related to cell kill when evaluating the potential of a given therapy for clinical application.

INFLUENCE OF ANALYTICAL FACTORS ON LIFE SPAN STUDIES

Cause of Death Analysis

Although this discussion has focused on the biological mechanisms that influence a brain tumor's response to therapy, data collection and data analysis can also have a profound influence on the conclusions. For example, in our experiments designed to measure increases in life span, the brain of every rat that dies or is killed is removed and sectioned, and the presence or absence of brain tumor is determined. Consequently, rats that die as a result of toxicity are never included as tumor deaths. This procedure is essential because toxicity controls in nontumor-bearing rats may not accurately predict toxicity in tumor-bearing rats. In addition, both of our 9L tumor sublines are invasive, and not only seed throughout the cerebrum but also into the cerebellum, brain stem, and spinal cord. Because our whole brain irradiations encompass only portions of the brain stem and none of the spinal cord, rats can be paralyzed and die from tumors growing in these regions. In the 9L model, brain stem and spinal cord tumor deaths occur most often 35–45 days after implantation. In our data analysis we assume that if ic tumor is present, the rat would have eventually died from the tumor; if it is absent, we assume that the rat is "cured".

If these brain stem and spinal cord tumors (> 50% of the deaths in some experiments) are not uniformly distributed among all the comparison groups, significant differences in interpretation can result. For example, in an experiment comparing the efficacy of various BCNU-radiation schedules, no rats died with spinal cord or brain stem tumors in the twice-weekly treatment group, whereas a number of them did in the thrice-weekly and five times a week groups (table 3). When Kaplan-Meier plots of these data were made on the basis of ic tumor-free survival (fig 1a), no statistical difference between the experimental groups was found ($P > 0.2$). However, when the plots were constructed on the basis of those that actually died of ic tumors (fig 1b), the twice-weekly treatment group was found to be significantly worse ($P < 0.05$). Approaches that include or exclude toxicity deaths from data can lead to similarly ambiguous conclusions. Consequently, nonparametric statistics that account for censored observations can minimize errors in interpretation only if the basic histological information for every rat is available.

Covariate Analysis

Covariate analysis of the mortality data with parameters that may be determinants of the time of death is also important. In our 9L brain tumor trials, we examined body weight at implantation, at initiation of treatment, and at the end of treatment; the position of the tumor; complications of implant (intraventricular involvement, sc tumors, subdural tumors, etc); and sickness grade (a modified Karnofsky scale of 0–+3) at the time of treatment and at the end of treatment. In studies designed to determine whether the interaction between BCNU and radiation had the same time dependence in situ as in vitro, only sickness grade and weight at the end of treatment were related to the time of death (14). The statistical analysis indicated a

TABLE 3.—Cause of death after treating 30–40-mg 9L tumors with various schedules of BCNU + radiation

Schedule	Ic tumor	Brain Stem or Spinal Cord Tumor		Toxicity	180 Day survivor
		With Ic tumor	Without Ic tumor		
5x/wk (10)*	1	3	2	2	2
3x/wk (10)	1	6	2		1
2x/wk (9)	5			2	2

*Number in parentheses = number of rats treated

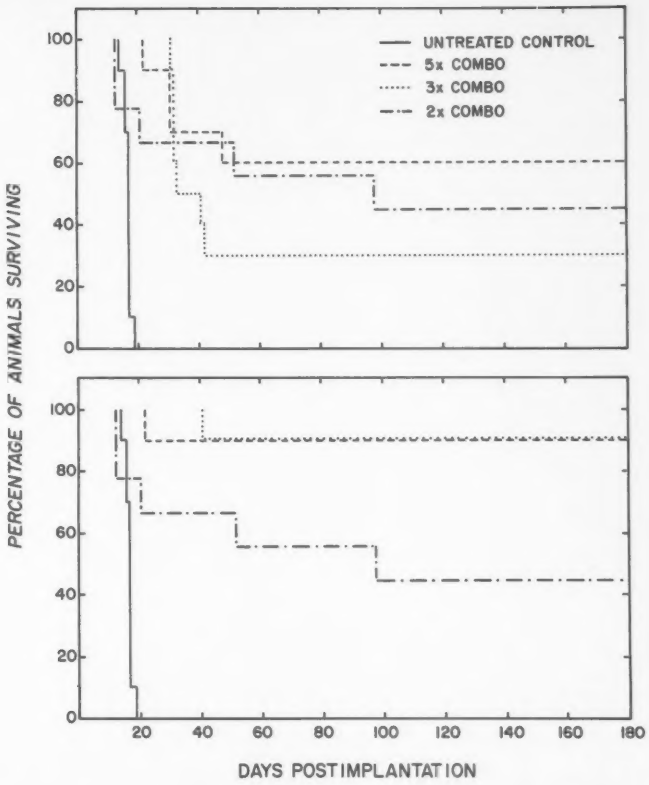

FIGURE 1.—Comparison of Kaplan-Meier plots for rats bearing 9L brain tumors after various treatments based on either intracerebral tumor-free survival (A), or death caused by intracerebral tumor (B). —— = untreated controls, - - - - - - = 2 mg/kg/day of BCNU 16 hr before 260 rads/day of x-rays, 5 x/wk for 2 weeks. ····· = 2.5 mg/kg/day of BCNU 16 hr before 433 rads/day of x-rays, 3x/wk for 2 weeks. —'— = 3 mg/kg/day of BCNU 16 hr before 520 rads/day of x-rays, 2x/wk for 2 weeks.

strong cross-correlation of sickness grade with animal weight, which is not surprising because weight loss certainly is associated with sickness. This experimental observation is identical to the clinical situation where, in general, brain tumor patients with higher Karnofsky ratings do better than patients with lower Karnofsky ratings.

At least one situation arose that may demonstrate the potential usefulness of covariate analysis for brain tumor therapy trials. Rats that completed a BCNU-alone treatment and had intraventricular tumor survived longer than those that had no intraventricular tumor. Because the presence or absence of intraventricular tumors is a function of both the implantation procedure and the subsequent tumor growth pattern, rats have more intraventricular tumors in some experiments than in others. Theoretically, BCNU or any other agent or therapy schedule could appear to be much more effective in one experiment than in another for reasons that may not be usefully translated into clinical trials. Covariate analysis will rapidly convince most investigators that rats should be randomized by weight into treatment groups after implantation and that all internal controls for therapy arms should be included in each study. After analysis of all these covariates we were able to determine within reasonable limits that there was a time-dependent interaction between BCNU and radiation in ic 9L tumors (14). This interaction was identical to that observed with cultured 9L cells (1). However, in the absence of a

covariate analysis no such compelling argument could have been made.

Statistical Analysis of Life Span Data

The last factor to be discussed that can influence the interpretation of brain tumor therapy data is the statistical analysis of animal survival data. We have compared the Wilcoxon-Gehan procedure (22), the Breslow modification of the generalized Wilcoxon procedure (23), and the Mantel (24) and Cox (25) modification of the generalized Savage procedure for analyzing data such as those in figure 1. Basically, the Wilcoxon-Gehan procedure weights all samples equally and accounts for single censored observations. The Breslow modification weights the early deaths more than the late deaths and accommodates censored observations. The Mantel-Cox modification weights the late deaths more than the early deaths and accommodates censored observations. As an example of the difficulties that can occur, two sets of data were analyzed by the latter two procedures (fig 2).

In the first case (fig 2a), the Breslow procedure indicated that the two therapies were not statistically different ($P > 0.2$), whereas the Mantel-Cox procedure indicated that they were statistically different ($P < 0.05$). In the second case (fig 2b), the Breslow procedure again indicated that the two therapies were not statistically different ($P > 0.05$), whereas the Mantel-Cox procedure indicated that they were statistically different ($P < 0.01$). Five additional cases were found among the 55 pairwise comparisons made in this experiment. Because several were less extreme cases, a visual judgment on the merits of the statistical test would be difficult. It is therefore essential that an investigator clearly and specifically define the aim of the study before selecting a statistical analysis. For example, for malignant brain tumors, where no effec-

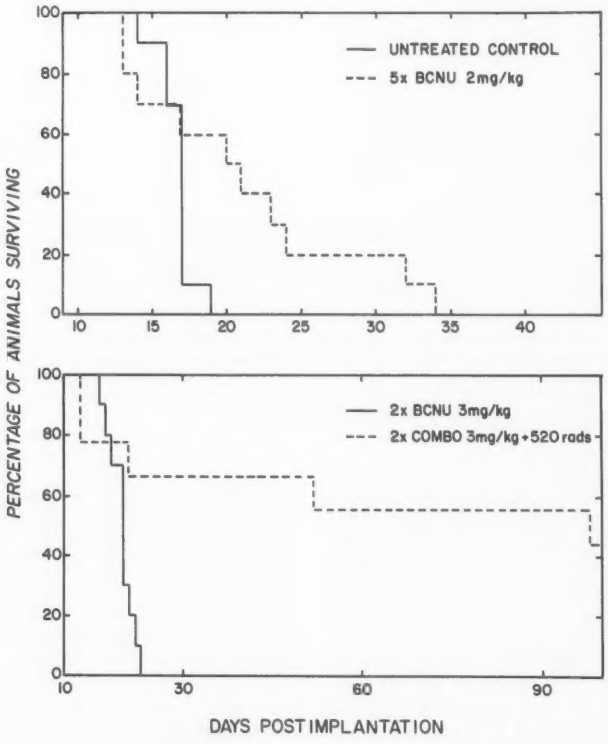

FIGURE 2.—Kaplan-Meier plots for rats bearing 9L brain tumors after various treatments. (A) —— = untreated controls, - - - - = 2 mg/kg/day of BCNU, 5x/wk for 2 weeks. (B) —— = 3 mg/kg/day of BCNU, 2x/wk for 2 weeks, ------ = 3 mg/kg/day of BCNU 16 hr before 520 rads/day of x-rays, 2x/wk for 2 weeks.

tive therapy exists, the appearance of a few long-term survivors may be very important. The Mantel-Cox analysis makes it more likely that this will be statistically demonstrated. However, in cases where reasonably effective therapies already exist, it may be important to avoid therapies in which a few early deaths occur. The Breslow analysis enhances the probability of statistically demonstrating this. Obviously, a clear understanding of the experimental objective is necessary to select the correct statistical analysis.

CONCLUSION

As experimentalists attempting to perform brain tumor research that may someday be relevant to the treatment of one of the most complex diseases known, our task is formidable. The usefulness of increased life span data from experimental brain tumor models is surely questionable unless the biological and analytical factors discussed here are adequately defined. Successful extrapolation to the clinic can only be achieved if we willingly accept this fact as a stimulating challenge to our physical and intellectual resources rather than as a constant source of frustration to be ignored whenever possible.

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Primary Intracranial Gliomas: Clinical Studies and Treatment Regimens of the Brain Tumor Research Center, University of California, San Francisco, 1977-1979[1]

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Four years ago, at our first Conference on Brain Tumor Therapy at Asilomar, participants were enthusiastic about the future of chemotherapy as an adjunct to surgery and radiation therapy. That initial enthusiasm has been dissipated by a discouraging experience shared by all of us. No new drugs that substantially improve on the nitrosoureas (BCNU, CCNU) or procarbazine have become available. Our group has evaluated to a limited extent the use of dianhydrogalactitol (DAG) against primary gliomas and medulloblastoma (1) and thio-TEPA against primary gliomas (2), neither of which was as effective as the nitrosoureas or procarbazine.

Our major effort has been directed toward developing criteria to evaluate tumor progression, determining the significance of the postirradiation "slump," evaluating initial diagnostic factors that might predict a better prognosis, and carrying out clinical studies incorporating cell cycle specific phase drugs into existing therapies based on nitrosoureas and procarbazine.

CRITERIA FOR EVALUATING TUMOR PROGRESSION

In 1977 we published criteria for evaluating brain tumor patients and described a numerical rating scale designed to designate tumor progression and tumor regression (3); the scale is based on sequential neurological examination, radionuclide (RN) brain scan, and computerized tomographic (CT) brain scan, done every 6–8 weeks before each treatment. Our conclusion was—and remains—that while each test has great accuracy, a combination of two of the three tests is more reliable for predicting tumor progression than any single test. The RN and CT scans are complementary. We have found that the RN scan is superior for demonstrating subarachnoid spread of tumor, but the CT scan is superior for demonstrating ependymal tumor spread; the CT scan is superior to the RN scan for determining the status of a patient with a cystic glioma, but the RN scan is superior to the CT scan for demonstrating early tumor transformation or progression of a highly infiltrative anaplastic astrocytoma. These are a few examples of areas in which the two studies can differ.

POSTIRRADIATION CNS DYSFUNCTION

Recently we used these criteria to evaluate the syndrome sometimes called the "postirradiation slump," characterized by increased focal or generalized central nervous system dysfunction in the early postirradiation period. The postirradiation slump does not appear to correlate with brain edema and increased brain capillary permeability (4,5) and probably reflects radiation-induced transient demyelination. Fifty-one patients harboring malignant gliomas who were randomized into a phase III study and had survived > 26 weeks from the time of diagnosis formed the basis of this study (6). The criteria for tumor progression were an unequivocal worsening of two of the three tests—neurological examination, CT scan, or RN brain scan. If changes occurred simultaneously at one examination or sequentially in two examination periods, 49% of the patients in this study would have been considered to have deteriorated from 0 to 18 weeks following radiation therapy. Of that group, 72% continued to show evidence of further tumor progression, while 28% subsequently improved without any change in chemotherapy regimen except for a short course of glucocorticoids during the postirradiation period.

In contrast, patients who showed no deterioration in one of the tests, or showed initial deterioration followed by improvement in that test in the next 16 weeks, had a median time to tumor progression (TTP) of 61–81 weeks; any patient who showed worsening during the first 18-week period had a 33% probability of significant improvement in a subsequent evaluation period. After 18 weeks, however, the probability of

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the test improving was only 1%. Thus if an effective alternative therapy were available to the patient in the postirradiation period, it should probably be offered when the above criteria show evidence of tumor progression.

COVARIATES INFLUENCING RESPONSE DETERMINED FROM CT SCANS

During the accumulation of data for the studies discussed above we decided to evaluate factors found on the routine nonenhanced and contrast enhanced CT scan taken after surgery but before initiation of either radiation therapy or chemotherapy, to determine if there were factors that might predict a better prognosis. Fifty-eight consecutive patients had sufficient CT scans to evaluate the extent of midline shift, peritumor low density, constant enhancement of tumor, and intratumor low density at the time of tumor progression (7). Midline shift, peritumor low density, and estimated volume of contrast enhanced tumor for patients surviving >26 weeks compared to those succumbing early are shown in table 1.

When these factors were used as covariates to determine their influence on the TTP (survival function), it was evident by both the Weibull or Cox methods that TTP was closely associated with tumor size and the presence or absence of peritumor low density. In fact, on the average, patients who showed no peritumor low density had a 40% chance of no tumor progression at 1 year, while there was a 60% probability of no progression if peritumor low density was seen on the CT scan before initiating radiation therapy. These differences become more marked for patients whose tumors are in the 90th percentile in terms of volume; the chances of no progression within 1 year were 10% without peritumor low density and 30% with peritumor low density. While it seems logical that the presence of a smaller tumor before the initiation of cytoreductive therapy is beneficial, there is no precedent for the benefit of peritumor low density, which is usually equated with edema; there was no difference in glucocorticoid dosage for these two groups of patients. We have tentatively attributed this to an enhanced peritumor leakage of immune competent cells, but there is no experimental evidence to support this supposition.

CLINICAL TRIALS

BHR-BR Therapy

First we evaluated the efficacy of hydroxyurea (HU) administered during radiation therapy and after BCNU to see if it improved the TTP of patients (8) with glioblastoma multiforme (GM) tumors and nonglioblastoma multiforme malignant gliomas (NGM; anaplastic astrocytoma, mixed malignant glioma, and gemistocytic astrocytoma).

The schemes used for this protocol are shown in table 2; table 3 lists patients with GM and NGM in the two arms of the study. By our criteria for tumor progression, only 15%–20% of GM patients showed a response to therapy, while 67% showed stability of disease for variable periods of time. Patients with glioblastoma multiforme, and particularly those who had a subtotal or total tumor resection and a Karnofsky rating of ≥ 60 at the time of study entry, seemed to benefit from the addition of hydroxyurea to BCNU and radiation therapy (BHR). Patients in the BCNU-radiation therapy (BR) arm had a median TTP of 31 weeks, while patients who received concomitant HU had a median time to tumor progression of 49 weeks. For GM patients receiving BCNU-radiation therapy alone, the likelihood of surviving 2 years was 0%, whereas the likelihood of surviving 2 years was 20% for those patients who received HU.

Another important finding of the study was that patients with NGM tumors, which were clearly malignant by all criteria, in general did considerably better than patients with GM tumors. The median TTP for all NGM patients (n = 38) was 66 weeks.

TABLE 1.—Comparison of CT measurements to time to tumor progression

	Average ± SE		
	Midline shift (mm)	Peritumor low density (diameter) (cc)*	Estimated volume of enhanced tumor (cc)*
48 patients continuing ≥ 26 weeks	6.2 ± 0.5 (n = 46)	60.8 ± 5.5	28.0 ± 4.3
10 patients progressing earlier	8.1 ± 1.4 (n = 8)	11.0 ± 4.5	74.6 ± 17.7
P, test of means	0.18	0.059	0.028

*Computations of volume assumed spherical coordinates, $V = \frac{4\pi}{3}$ (radius)^3.

TABLE 2.—Doses and schedules used in the treatment of malignant gliomas at Brain Tumor Research Center (UCSF) Study eponyms

	BHR	BR	BCNU-5-FU	PCV 3	BFHM	PFHM	Cycle therapy		
							One	Two	Three
Irradiation (RT)									
whole brain	5000 r	5000 r							
tumor bed	6000 r	6000 r							
BCNU (mg/m²)	80, d 1-3*	80, d 1-3	180, d 1		180, d 1				180, d 1
CCNU (mg/m²)				1i0, d 1				110, d 1	
Procarbazine (mg/m²)				60, d 8-21		150, d 1-14	150, d 1-28	60, d 7-21	
Hydroxyurea (mg/m²)	275, q 6h† qod‡ during RT only				400, q 6h d 24-26	400, q 6h d 24-26			
5-FU§ (g/m²)			1, d 15-17		1, d 15-17	1, d 15-17			1, d 15-17
6-Mercaptopurine (mg/m²)					100, q 6h d 33-35	100, q 6h d 33-35			
Vincristine (mg/m²)				1.4, d 8,29					
Cycle (seeks)	6-8	6-8	6-7	6-7	7	9	8	6	6-7

*d = day of cycle.
†q 6h = every 6 hours.
‡qod = every other day.
§5-FU given by continuous iv at 1 g/m²/day.

TABLE 3.—Glioblastoma multiforme patients

	BR	BHR
Total patients	26	35
Total or subtotal tumor resection	20	30
Clear response to therapy	15%	20%
Median time to tumor progression		
All patients	31 weeks	42 weeks
Total or subtotal tumor resection and Karnofsky performance score ≥ 60	31 weeks	49 weeks

Combination Therapy with 5-FU and BCNU

Data obtained by treating intracranial (ic) tumor bearing rats with 5-FU after they had received BCNU (9) suggested that use of 5-FU during the proliferation period following BCNU therapy increased long-term survival. By extrapolation of animal data to humans, we designed the clinical scheme shown in table 2(10). Of the 37 patients on this study, 29 were fully evaluable and were divided into GM and NGM groups. All patients had failed previous irradiation and had evidence of tumor progression at least 3 months after the completion of radiation therapy.

The BCNU-5-FU program was reasonably successful. The combination temporarily arrested disease progression in 83% of patients, although the absolute response rate was 1/4 for GM and 8/25 for NGM. Patients in the NGM and GM group who responded to therapy tolerated higher doses of BCNU for the first five courses than patients who were stable only on this treatment. Retrospective comparison of this program with previous programs with BCNU-procarbazine (11) or BCNU alone (12) indicated that the long-term prognosis for these patients with recurrent tumors was unchanged as a result of BCNU-5-FU. However, there was an improvement in median survival.

Combination Therapy with CCNU, Procarbazine, and Vincristine

After the BCNU-5-FU study was closed, we analyzed the effect of the change in treatment schedule and dose for the combination of CCNU, procarbazine, and vincristine (PCV 3) for patients with recurrent malignant gliomas (13). Of 46 patients with recurrent malignant gliomas treated on this program, 12 harbored GM and the remaining 34 harbored NGM tumors. Because 27 of these 46 patients had received prior chemotherapy, analysis of TTP was compared separately for patients who received PCV 3 as a second form of therapy and for patients who received PCV 3 as the treatment initiated after radiation therapy failed to arrest tumor progression. Patients who had not received chemotherapy showed a 42% response rate and a 42% rate of disease stability, whereas patients who had received prior chemotherapy showed only a 15% response rate and a 30% stabilization of disease. Interestingly, if a patient showed response or disease stabilization, the median TTP differed little among the groups that did or did not receive prior chemotherapy. Invariably, in both the PCV 3 and BCNU-5-FU studies, GM patients did considerably worse than NGM patients, with median TTPs of approximately 12–14 weeks compared to 26–29 weeks respectively (14).

For both BCNU-5-FU and PCV 3, the ability to stop disease progression seems to have been the most important common feature, but prolonged remission was not achieved by the new programs compared, respectively, to either BCNU alone or to PCV 1.

New Protocols

With the hope of improving long-term response we increased the number and scope of cell cycle phase specific (CCS) drugs used with BCNU for the treatment of recurrent malignant gliomas. A new protocol, BFHM, was developed using BCNU followed by 5-FU, HU, and 6-mercaptopurine (6-MP). The dosages and timing of therapy are listed in table 2. This program is ongoing and has accrued 24 patients to date. Figure 1 is the Kaplan-Meier curve for the TTP for the NGM patients who received no prior chemotherapy, all NGM patients whether they received prior chemotherapy or not, and all GM patients. At this point the results indicate that, at least for GM patients, there is little difference between this program and BCNU-5-FU or PCV3. The impact on NGM patients seems to be negligible, although the possibility of long-term response must be evaluated after accrual of more patients and longer followup.

This program is also being adapted for patients who failed nitrosoureas, to whom procarbazine is given at a dose of 100 mg/m² for 14 days in place of BCNU. The other innovation in this program is that if a patient remains myelotoxic for over 1 week longer than the designated length of the cycle, the cycle is begun again without the nitrosourea or procarbazine. Under these circumstances, toxicity has not been a problem and, in fact, two patients have actually shown a response as determined by improvement by both the neurological examination and CT or RN scan during the period they received 5-FU, HU, and 6-MP alone.

Another program currently under study is called "Cycle"; the scheme is listed in table 2. It is being used as an adjuvant form of chemotherapy following surgery and radiation therapy and for patients with recurrent tumors. The program incorporates three separate forms of therapy that have shown activity at our institution. Therapies are rotated to reduce the likelihood that cells will become resistant to one or more of the agents. The survival times and TTP for patients on the study are summarized in table 4. The

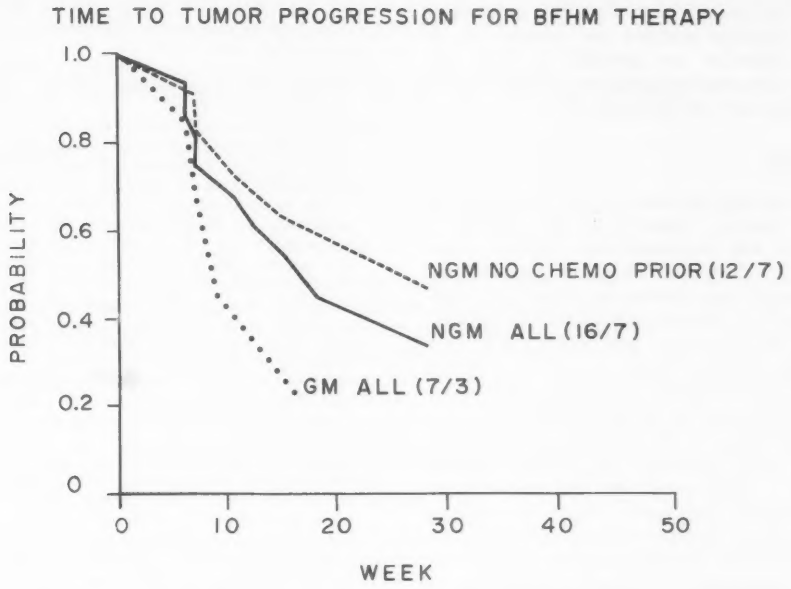

FIGURE 1.—Kaplan-Meier representation for time to tumor progression. GM = glioblastoma multiforme; NGM = nonglioblastoma malignant gliomas; number in parentheses = number in group/number censored.

TABLE 4.—Survival and time to tumor progression of patients on cycle therapy*

Age range	Mean KPS†	Survival (weeks)	TTP (weeks)
GM			
Adjuvant to radiation therapy			
7–54	85 ± 13	32, 23, 60+, 70+, 107+, 124S	9D, 14D, 47S, +65R, +107S, +124S
Primary therapy for recurrence			
54–72	80 ± 8	21+, 22, 53	+17S, 22S, 38S
NGM			
Adjuvant to radiation therapy			
27–45	90 ± 6	+31, +29, +83, +78, +95	17S, +28S, +70R, +76R +93S
Primary therapy for recurrence			
13–55	90 ± 10	+21, +19, 15, +21, 94, 56, +40, 118, +50, 106, +52, +105	+6S, +13S, 15D, +21S, 23S, 24R, +29S, 30S, +44R, 48S, +52S, +94S

*As of 1 March, 1981.
†Karnofsky Performance Score.
S = stable disease.
R = responder.
D = deteriorated throughout therapy.

age range of the patients in this study is quite broad and, clearly, younger patients are expected to do better. It is therefore not possible for us to say unequivocally how good this program may become, but data collected to date are encouraging.

CONCLUSION

We have developed reliable methods for evaluating response to therapy, quantifying the number of patients who will spontaneously improve after demonstrating evidence of CNS dysfunction following radiation therapy, and determining that peritumor edema is a prognostic factor in patients with malignant glial tumors. Unfortunately, our clinical trials have produced little progress. While we have been able to increase the number of patients who show response or stabilization of disease at the time of recurrence, we have had little impact on the ultimate and long-term prognosis of these patients. Our major problem remains a lack of effective and selective agents for the treatment of primary malignant gliomas. It is unlikely that new and effective agents will become available in the immediate future. The development of effective agents is a challenge that the participants of this conference must face.

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A Review of Studies of the EORTC Brain Tumor Group[1]

J. Hildebrand[2,*]

SUMMARY

The results of three randomized trials performed by the EORTC Brain Tumor Group in patients with histologically proven brain gliomas are presented. CCNU (130 mg/m² every 6 weeks) in combination with dexamethazone (4–20 mg daily) prolonged total survival compared to a group of patients treated only with corticosteroids. CCNU (130 mg/m² every 6 weeks) either alone or one day after VM-26 (100 mg/m²) produced an objective remission in less than 30% of treated patients. However, the two treatment modalities did not prolong the interval between surgical removal of the tumor and relapse (recurrence). None of 12 patients with signs of brain tumor recurrence previously treated with VM-26 plus CCNU responded to procarbazine (3-week courses of 150 mg/m²/day).

[Cancer Treat Rep 65 (Suppl 2):89–94, 1981]

Four parameters may be measured in trials testing chemotherapy in brain tumors: (*a*) prolongation of the survival time; (*b*) rate and length of objective remissions; (*c*) prolongation of the symptomatic "tumor-free interval" (FI); and (*d*) the number of long-time survivors. The respective advantages and drawbacks of these criteria are summarized in table 1. As in other neoplasias, these parameters do not necessarily measure the same phenomenon in patients harboring brain tumors and may give contradictory results, especially when the drugs used are only moderately active.

It has been the policy of the EORTC Brain Tumor Group to evaluate these parameters separately. An effort has been made to evaluate the length of the FI in particular, not only because such trials measure the quality of the survival, but also because supportive care is minimal during the FI and does not interfere substantially with the effects of chemotherapy. It is fully realized, however, that for patients harboring gliomas, the FI does not correspond to a disease-free period.

This report reviews the data of three EORTC Brain Tumor Group trials and compares their results with those reported by others. All patients studied had histologically-proven malignant hemispheric gliomas, were > 16 years old, and had an expected survival of at least 2 months.

The schedules of these three trials are shown in figure 1. In trials 26741 and 26751, patients considered for FI analysis did not require postoperative administration of dexamethazone and had normal or minimally abnormal neurological examinations. Patients entered into trial 26742 required steroid administration and/or had neurological signs difficult to quantify by a clinical examination.

RESULTS AND DISCUSSION

Effects of CCNU on the Duration of Survival Time

The effect of CCNU on the median survival time was estimated only in one trial (26742, fig 1). The survival time was significantly prolonged ($P < 0.01$; 31 weeks) in patients receiving CCNU (130 mg/m² every 6 weeks), compared to patients in the control group who did not receive chemotherapy (21.5 weeks). All patients received 5500–6000 rad cranial irradiation postoperatively and 4–20 mg/day of dexamethazone.

The number of patients included in the trial is small and its conclusion conflicts with the findings of Reagan et al (1), who failed to show a significant increase of the survival when CCNU was used in a randomized trial in combination with surgery and radiation therapy. When combined with radiation therapy, BCNU significantly prolongs the survival of patients with gliomas (2,3).

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TABLE 1.—Types of trials evaluating chemotherapy in malignant brain tumors

Measured parameter	Advantages	Difficulties	Types of patients
Survival time	Short constant (median: 24 weeks), easy to measure	Requires prolonged hospitalization	All patients
Objective remission	Hospitalization not required	More difficult to measure than ST	Recurring primary tumor
"Free interval"	Measures the quality of survival	Difficult to measure	Selected patients (with minimal neurologic deficit)

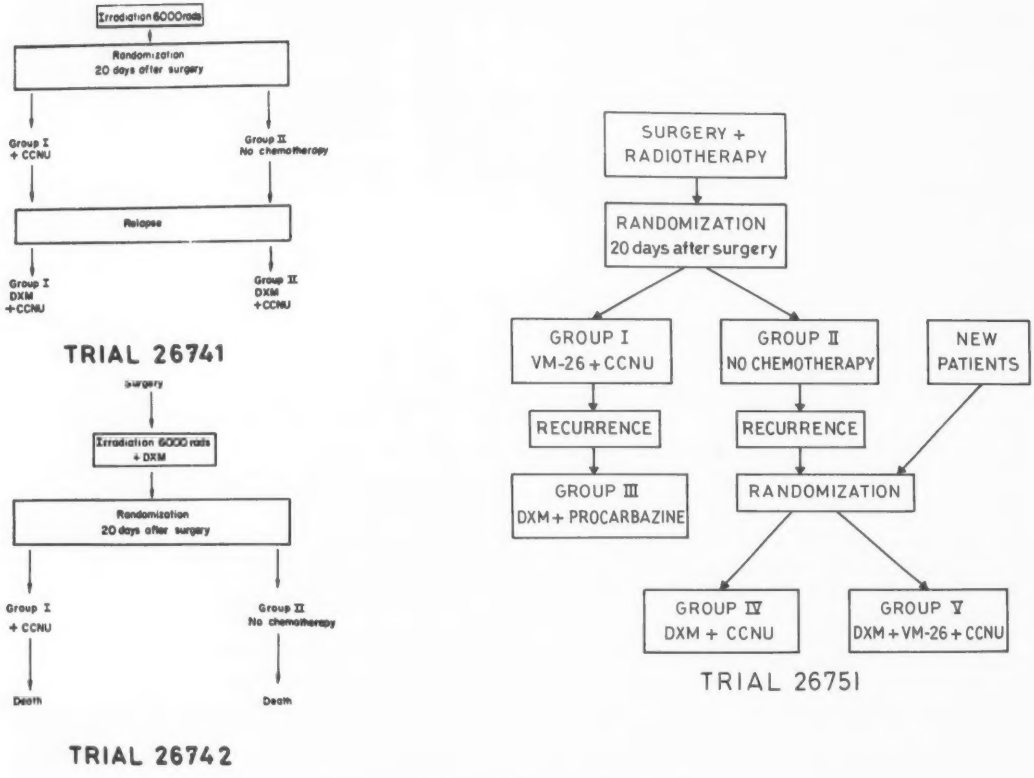

FIGURE 1.—Schedule of trials 26741, 26742, and 26751.

Effects of CCNU and VM-26 Plus CCNU or Procarbazine on the Rate and Duration of Objective Remissions

This parameter was evaluated in patients with symptoms and signs of neurological relapse who were previously operated and irradiated. Objective remission (OR) was defined as a clear-cut clinical improvement that lasted at least 6 weeks after complete discontinuation of steroids. In our study, stabilization of neurological status after a recent deterioration was not considered as an objective remission.

Effects of CCNU.—The effects of CCNU (130 mg/m² every 6 weeks) on the rate of OR were evaluated in 27

patients in trial 26741 (group II, fig 1) and in 14 patients in trial 26741 (group IV, fig 1). The rates of OR were 29% and 25%, respectively, with a median duration of +7 months.

VM-26 plus CCNU.—In trial 26751 (group V, fig 2), VM-26 (100 mg/m²) was administered 24 hours before CCNU (130 mg/m² every 6 weeks). OR was observed in 19% of 21 evaluable patients.

Procarbazine (PCZ).—PCZ was given at a daily dosage of 150 mg/m² in 21-day courses to 12 patients with neurological signs of recurrence (group III, fig 1). All patients had previously received the VM-26 plus CCNU combination until relapse. No OR has been observed to date.

The results are less encouraging than most of the data observed until now with various nitrosourea derivatives (CCNU, BCNU, and MeCCNU) and procarbazine (4). From the literature, comparable ORs for CCNU and 44% (5), 37% (6), and 24% (1). Only the last OR is comparable to ours. The discrepancies may be due to the fact that in EORTC studies, a delay of 6 weeks after complete discontinuation of dexamethazone is required to demonstrate an OR, and this may neglect short remissions. In addition, stabilization of the neurological status in patients with signs of tumor progression was not considered as OR in our trials.

Effects of CCNU or VM-26 plus CCNU on the Duration of the FI

The FI, defined as the period between surgery and a decline in neurological status, was measured in selected patients in whom the postoperative neurological deficit was absent or quantitatively measurable and who did not require the administration of corticosteroids after operation.

Effect of CCNU.—The effect of CCNU (130 mg/m² every 6 weeks; the mean dosage of CCNU actually given was at least 80% of the scheduled dose) on the length of the FI was evaluated in 81 patients (trial 26741). Its mean duration (fig 2) was not significantly prolonged in patients receiving chemotherapy (34.5 weeks) compared with the control group (31 weeks).

VM-26 plus CCNU.—The combination of CCNU (130 mg/m² every 6 weeks) given 1 day after VM-26 (100 mg/m²) has been evaluated so far in 104 patients in trial 26751. The difference between treated patients (median of 50 weeks) and controls (median of 30 weeks) is not significant with these data (fig 3). The study has not been closed.

To the best of our knowledge, the effects of CCNU on the FI (median time to progression) were reported in only one study, where the FI was prolonged in patients

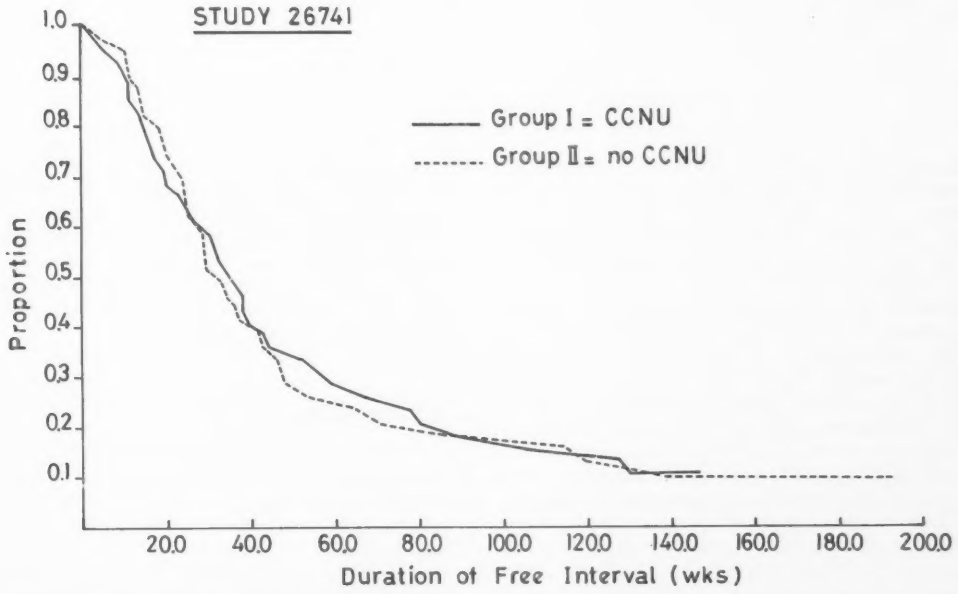

FIGURE 2.—Study 26741. Free interval length curves for 42 patients receiving CCNU (————) and for 39 control group patients (- - - - - - - - - - - - - -).

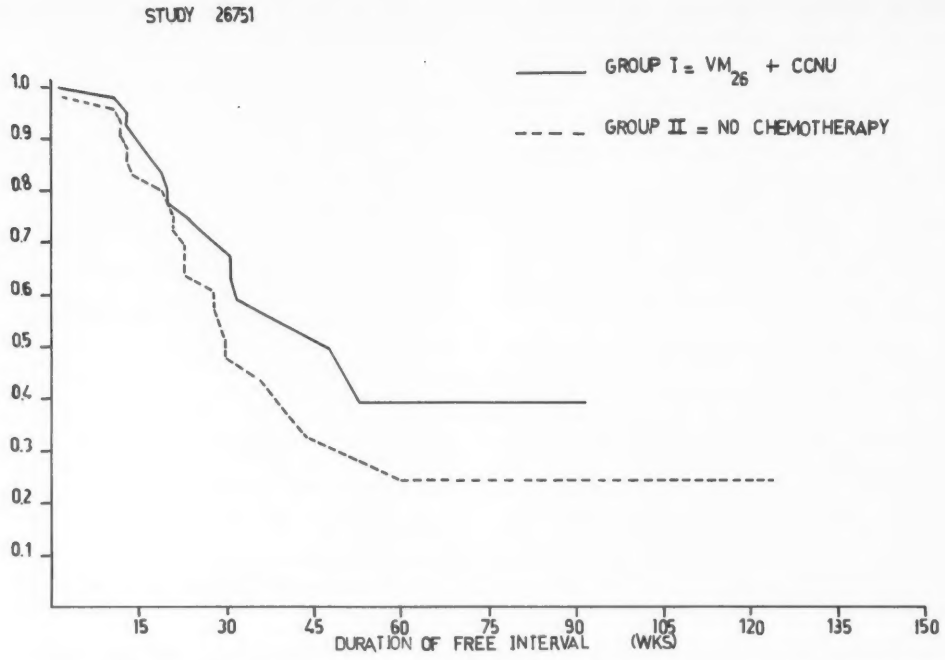

FIGURE 3.—Study 26751. Free interval length curves for 54 patients receiving VM-26 + CCNU (————) and 50 control group patients (- - - - - - - - - - - - - - -).

who received CCNU (7). The discrepancy between these results and our data are difficult to explain. Scheduled doses of CCNU were the same in both studies. In our trial, no patients received dexamethazone before recurrence, and the number of patients studied was larger than previously reported (7).

When considered in the whole group of 81 patients, regardless of chemotherapy in trial 26741, the FI was longer for patients < 50 years of age (median duration 60.2 weeks) than in older patients (28.6 weeks) (fig 4). This interval tended to be shorter in patients with glioblastoma multiforme (29 weeks), but the difference was not statistically significant. No differences in the length of the FI were found by comparing males to females, gross total tumor resections to lesser operations (subtotal resection or biopsy), or patients with neoplastic involvement of one lobe to patients with lesions extending to more than one lobe. Evaluation of prognostic factors in the second trial (26751) gave the same results. Age was the major prognostic factor.

In trial 26741, the total survival time of patients who received CCNU after relapse tended to be longer (62 weeks) than for patients treated within 3 weeks of surgery (43 weeks) ($Q = 0.07$). Survival measured from relapse (fig 5), was longer for patients treated only after recurrence (22.5 weeks) compared to the early treatment group ($P = 0.02$). From our data, we recommend that when used alone CCNU be given only after the relapse of neurological signs.

CONCLUSION

The combined results of these trials show that CCNU or the combination of VM-26 and CCNU can produce objective remissions in patients harboring malignant gliomas who demonstrate signs of recurrence. They are ineffective as adjuvant chemotherapy and, so far, do not seem to prolong the free interval. CCNU given alone seems to prolong the total survival time in patients treated by radiation therapy and with corticosteroids.

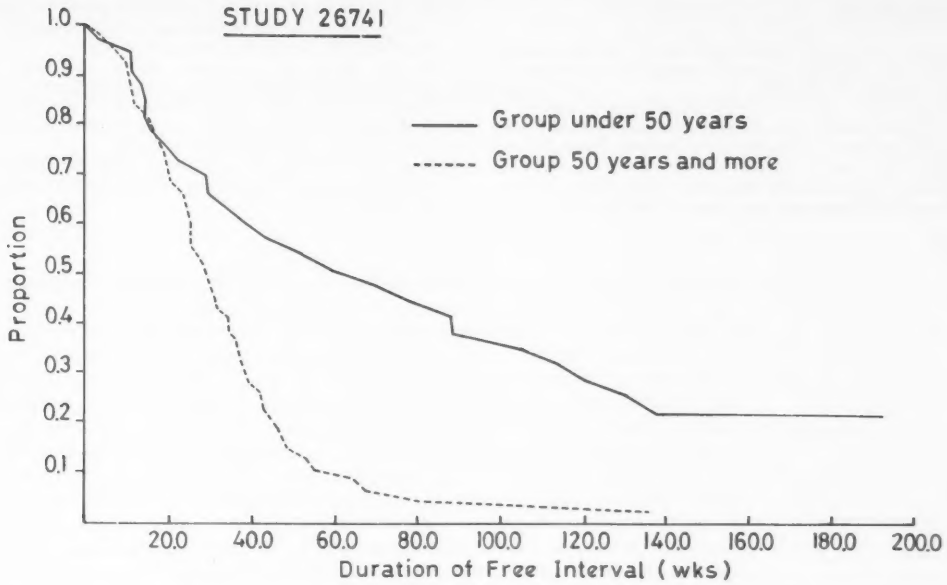

FIGURE 4.—Study 26741. Free interval length curves for 33 patients under age 50 (————) and for 48 patients aged 50 years or more (- - - - - - - - - - - - - - -).

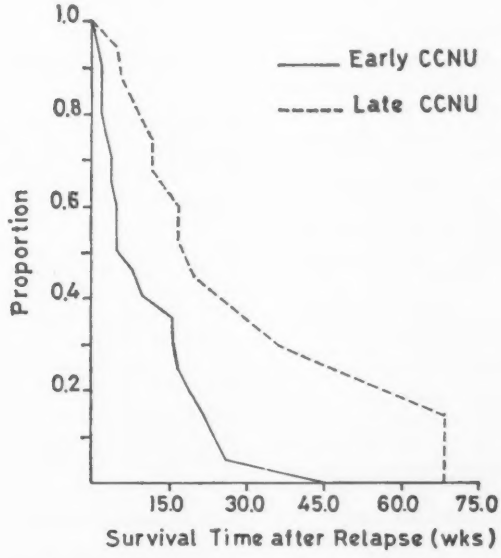

FIGURE 5.—Study 26741. Survival time curves after relapse for 24 patients treated with CCNU 3 weeks after surgery (————) and for 34 patients treated with CCNU only after relapse (- - - - - - - - - - - - - - -).

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Radiosensitizers: Rationale and Potential[1]

J. Martin Brown[2,*]

SUMMARY

This paper briefly reviews agents that are capable of sensitizing hypoxic cells to radiation and chemotherapeutic agents. The first part is a synopsis of the development of hypoxic radiosensitizers, which concludes that misonidazole can be effective against human tumors. Unfortunately, neurotoxicity limits its effectiveness in humans because the dose that can be given in conjunction with daily fractionated radiation is five to ten times lower than is required for full radiosensitization of the hypoxic cells. The second part covers our recent efforts to develop a drug that does not produce such limiting neurotoxicity. The primary rationale of our program was to synthesize a drug with a short plasma half-life that was too hydrophilic to cross the blood-brain barrier but was able to penetrate tumors and radiosensitize hypoxic cells. From this program, a new drug, SR-2508, has been found that is as efficient as misonidazole in its radiosensitizing ability, but is four to ten times less toxic. Finally, the potential of radiosensitizers not only as agents that can sensitize tumor cells to radiation, but also as agents that can specifically sensitize tumors to chemotherapeutic agents, is discussed. In addition, these drugs may be potential cytotoxic agents that produce toxicity only in solid tumors.

[Cancer Treat Rep 65 (Suppl 2):95–102, 1981]

DEVELOPMENT AND CURRENT USE OF RADIOSENSITIZERS

The classic work of Gray and his colleagues (1) demonstrated the universality of the oxygen effect using a variety of different endpoints with a variety of different biological systems. They demonstrated that under hyperbaric conditions radiation was more cytotoxic than the same radiation dose given under air breathing conditions, and stressed the possible importance of these findings to radiation therapy. However, it was the histological study done 2 years later by Thomlinson and Gray (2) that convinced most radiotherapists and radiobiologists that human tumors probably contained hypoxic, radioresistant cells. This study first proposed the now classic model of hypoxia in tumors. According to this model, hypoxic cells in tumors occur at a distance of 100–150 μ from blood vessels and border regions of necrosis. Because they are resistant to radiation-induced cell kill, hypoxic cells would survive treatment with radiation and would eventually repopulate the tumor.

In 1963 Adams and Dewey (3) introduced the important concept that the electron affinity of a compound determines its ability to radiosensitize hypoxic cells. In the mechanism of action of electron affinic agents, radiation produces free radicals that react rapidly with electron affinic compounds to form irreparable adducts.

However, although several agents were found that radiosensitized hypoxic bacterial cells, for a long time none were found that radiosensitized mammalian cells. Then it was found that *p*-nitroacetophenone (PNAP) could radiosensitize both hypoxic bacterial cells and mammalian cells at nontoxic doses (4), and a study of analogs of PNAP established the importance of the nitro group on the benzene ring for this mode of action (5). Further work by Chapman and his colleagues (6) demonstrated the importance of the nitrofurans as radiosensitizers. Some nitrofurans can radiosensitize hypoxic mammalian cells as much as oxygen, but they do not radiosensitize animal tumors in vivo. However, the structurally similar but slightly less electron

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affinic nitroimidazoles do radiosensitize hypoxic cells in animal tumors. The first demonstration of this was with the 5-nitroimidazole (metronidazole, Flagyl); it was soon demonstrated that the 2-nitroimidazole (Ro-07-0582, misonidazole) was a more effective agent (7,8).

Table 1 lists data from our laboratory that illustrate the degree of radiosensitization (or the enhancement ratio) that can be obtained when misonidazole is injected before tumor-bearing animals are irradiated. The endpoint was the dose to cure 50% of the animals of their leg-implanted tumors (TCD_{50}). With a large dose of misonidazole (1 mg/g), the TCD_{50} is reduced from 7125 rad to 3100 rad for an enhancement ratio of 2.30. Almost all of this is pure radiosensitization, although there is a small component due to the direct cytotoxic effect of the drug. At the lower dose (0.3 mg/g) of misonidazole, the enhancement ratio is still greater than 1.8, and all of this effect is due to radiosensitization.

These data were obtained with large, single radiation doses. Because reoxygenation between doses is likely (9), a lower enhancement ratio for fractionated doses would be predicted; as listed in table 1, this was found. Nevertheless, a factor of 1.4, which is equivalent to 40% more radiation dose to the tumor cells for no additional radiation dose to the surrounding normal tissue, would be of great benefit in radiotherapy.

Both metronidazole (10) and misonidazole (11) can radiosensitize human tumors. However, it has become apparent that the neurotixicity of misonidazole limits levels of the drug in tumors to below values that are optimum for radiosensitization. In fact, when misonidazole is given before each dose in a conventional, fractionated regime, the development of neuropathy (both peripheral and central) limits tumor concentrations of the drug to approximately 20 μg/g. At this concentration, enhancement ratios would not be expected to be greater than 1.3. Thus it is clear that misonidazole is not the optimum hypoxic cell radiosensitizer for clinical application. Such a compound should be as effective as misonidazole in its radiosensitizing ability, but should be only one fifth to one tenth as toxic.

DEVELOPMENT OF NEW RADIOSENSITIZERS

A replacement for misonidazole can in principle be produced in two different ways. First, the new drug could be a more efficient radiosensitizer (ie, give the same enhancement ratio at a lower concentration) and be as toxic as misonidazole. Alternatively, the new compound could be equal to misonidazole in its radiosensitizing ability but be less toxic, which allows more drug to be given to achieve the same enhancement ratio. Over the past 2 years we have been exploring the latter possibility by modifying the pharmacokinetic properties of the drugs.

The basis for modification is shown in figure 1. Two possibilities exist. First, because the degree of radiosensitization achieved by a given drug depends only on the concentration of drug in the hypoxic cells at the time of irradiation, and toxicity is likely to be a function of the area under the curve (AUC) of drug concentration versus time (12), it follows that a drug with a shorter plasma half-life than misonidazole would be superior to misonidazole, provided it had an undiminished peak concentration in the tumor. Second, it might be possible to exclude the drug selectively from tissues that limit the drug dose. Because both central and peripheral neurotoxicity is the dose-limiting toxicity in humans, it follows that a radiosensitizer that is excluded from neural tissue but is maintained at undiminished levels in the tumor should be superior to misonidazole for clinical use.

The problem is to design a 2-nitroimidazole analog of misonidazole that has a shorter half-life and is partially excluded from neural tissue. In general, the lipophilicity of pharmacological agents has a major influence on their activity, half-life, distribution, and toxicity (13,14). In particular the work of Soloway and colleagues (15,16) has shown that lipophilicity is the major determinant of a drug's ability to penetrate the blood-brain barrier. Thus, it seems reasonable to expect that the two methods of reducing toxicity shown in figure 1 might be achieved by modifying the lipophilicity of the drugs. This can be achieved by substituting different side chain groups at the 1-position of the 2-nitroimidazole ring.

TABLE 1.—Effect of misonidazole (MIS) on the TCD_{50} of the MDAH/MCa4 mammary carcinoma

Drug and dose injected*	Number of fractions	TCD_{50} (rad) (95% confidence interval)	Enhancement ratio (95% confidence interval)
Saline	1	7125 (6600–7700)	—
MIS (0.3 mg/g)	1	< 4000	> 1.8
MIS (1.0 mg/g)	1	3100 (2960–3250)	2.30 (2.10–2.51)
Saline	10†	9700 (8600–10860)	—
MIS (0.3 mg/g)	10†	7000 (6730–7200)	1.39 (1.23–1.56)

*Injected ip 30 minutes before the start of the radiation exposure.
†Ten equal doses given daily (ie, in 9 days) with saline or MIS 30 minutes before each exposure.

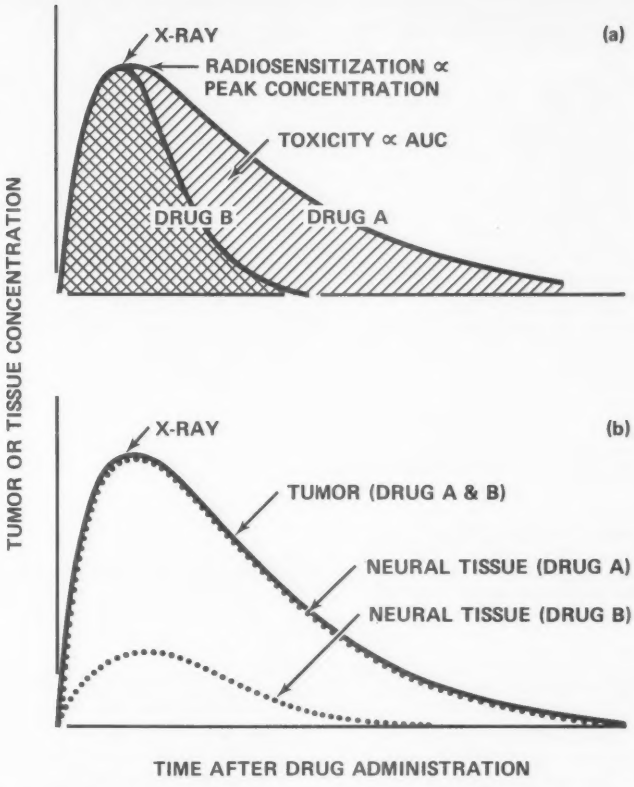


FIGURE 1.—Two methods of reducing the neurotoxicity of a radiosensitizer without affecting its ability to radiosensitize the tumor. Both are based on the fact that the radiosensitization depends on the concentration of sensitizer in the tumor at the time of irradiation, whereas toxicity is determined by the total tissue exposure, or area under the curve (AUC) of drug concentration versus time. Panel (a) shows that drug B, which has a shorter plasma half-life than drug A, yet achieves the same peak concentration, should be superior to drug A. Panel (b) shows the possibility that drug B should be less neurotoxic than drug A if it can be selectively excluded from neural tissues compared to the tumor.

Thus, with the expectation that the degree of lipophilicity would influence the distribution and concentration of radiosensitizers in various tissues without affecting their radiosensitizing efficiency, we selected ten 2-nitroimidazole analogs of misonidazole that covered a 100-fold range of partition coefficients (0.014 to 1.5) and determined for these compounds the acute LD_{50} in BALB/c mice, plasma half-lives, tumor and brain penetration, and their ability to radiosensitize EMT6 tumors in vivo.

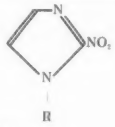
Table 2 lists the structures, partition coefficients, one-electron reduction potentials (electron affinities), and acute LD_{50} values of the ten 2-nitroimidazoles used in the present study. Compounds prefixed "Ro" were provided by Roche Laboratories, Welwyn Garden City, UK, those prefixed with "SR" were synthesized by Dr. William W. Lee, SRI International, Menlo Park, CA.

For each of the ten 2-nitroimidazoles, we determined the concentration as a function of time after a single ip injection of the drug in plasma, tumor, and brain of BALB/c mice bearing the EMT6/Ca/VJAC tumor implanted sc in the flank. Tumor/plasma ratios reached a maximum at 30–45 minutes after injection and thereafter were maintained at the same level (ie, the tumor and plasma concentration versus time curves were parallel). This was also true of brain/plasma levels

TABLE 2.—A listing of the 2-nitroimidazoles used together with some of the physical and biological properties

	Compound			Partition coefficient		Acute LD_{50}
	Designation	Structure (R)*	Mol wt	(P)†	E_7^1(mV)	(mg/g)‡
1	Ro-07-0269	$-CH_2CHOHCH_2Cl$	205	1.5§	−384§	~0.18
2	Rp-07-0913	$-CH_2CHOHCH_2OEt$	215	1.27¶	−391**	>1.1
3	SR-2514	$-CH_2CH_2NO.HCl$	263	0.82	−390**	~0.9
4	Ro-07-0741	$-CH_2CHOHCH_2F$	189	0.44	−383**	~0.9
5	Misonidazole	$-CH_2CHOHCH_2OMe$	201	0.43	−389††	1.8 (1.3–2.6)
6	Ro-05-9963	$-CH_2CHOHCH_2OH$	187	0.13	−389††	3.1 (2.2–4.4)
7	SR-2512	$-CH_2CH_2SOCH_3$	203	0.060	ND‡‡	~2.0
8	SR-2508	$-CH_2CONHCH_2CH_2OH$	214	0.046	−388§§	4.9 (4.3–5.6)
9	SR-2555	$-CH_2CON(CH_2CH_2OH)_2$	258	0.026	−398§§	>7.7
10	SR-2530	$-CH_2CONHCH_2CHOHCH_2OH$	244	0.014	−392§§	>6.1

*Structure of the side chain (R) at the 1-position of the 2-nitroimidazole ring



†The partition coefficient (P) in octanol/water was determined using the method of Ref 17.
‡$LD_{50/2d}$ (with 95% confidence intervals) for BALB/c mice weighing 20–25 g.
§Ref 18.
¶Ref 19.
**P.D. Clarke and P. Wardman, personal communication.
††Ref 20.
‡‡Not done due to insufficient drug. Based on its structure and radiosensitizing efficiency its E_7^1 would be expected to be similar to that of MIS.
§§P. O'Neill and S. Ho, personal communication.

for the more lipophilic compounds ($P \geq 0.44$), but for the hydrophilic drugs the brain/plasma ratio tended to rise during the 135-minute measurement period.

Figure 2 shows the mean tumor/plasma and brain/plasma ratios for the ten 2-nitroimidazole radiosensitizers as a function of their partition coefficients. The tumor/plasma ratio for each compound is the mean value from all mice killed at and beyond the time of the peak tumor/plasma ratio. The brain/plasma ratio is the mean value from all the mice for each compound. It can be seen from figure 2 that the tumor/plasma ratio is largely independent of partition coefficient over almost the entire range studied (from $P = 0.026$ to 1.5). There is, however, a suggestion of a fall in this ratio for the most hydrophilic compound, SR-2530. On the other hand, the brain/plasma ratio shows a marked dependence on lipophilicity at partition coefficients below approximately 0.4. At values above this up to a partition coefficient of 1.5, the brain/plasma ratio is 90%-100% and is independent of partition coefficient.

Figure 3 shows the tumor/brain ratio as a function of partition coefficient. The mean value of this ratio for compounds with a partition coefficient equal to and greater than misonidazole is 0.9. As the lipophilicity of the compounds decreases, however, the tumor/brain ratio increases steadily and reaches a value of 13.5 ± 1.3 for SR-2555 and some value greater than this for the most hydrophilic compound, SR-2530.

Thus it is clear both from the acute toxicity studies (table 2) and from the tumor/brain ratios that the more hydrophilic compounds, SR-2508, SR-2555, and SR-2530, would seem to be excellent candidates to replace misonidazole. However, are they as good as misonidazole at radiosensitizing tumors in vivo?

Figure 4 shows the cell survival of EMT6/St/lu tumors implanted in mice that were irradiated with 2000 rad at different times after an iv injection of either SR-2508 or SR-2555. Both drugs were injected at a dose of 2 mmol/kg. The upper panel shows the tumor concentrations of SR-2508 and SR-2555 obtained in the same tumors used to assay for cell survival (one-half of each tumor was used to assay for tissue levels, and the other half was used to assay for cell survival). As can be seen from this experiment lower levels of cell survival were obtained with SR-2508 plus radiation than with SR-2555 plus radiation. Since neither drug produces any tumor cell killing in the absence of radiation, this means that SR-2508 is a better radiosensitizer than SR-2555 (since both give similar levels of drug concentration in the tumor). In other similar experiments we found that SR-2508 was equally as effective as misonidazole as a radiosensitizer.

Figure 5 shows the data from a similar experiment performed with the more hydrophilic drug SR-2530. Despite achieving tumor levels of 1–2 mM in the first

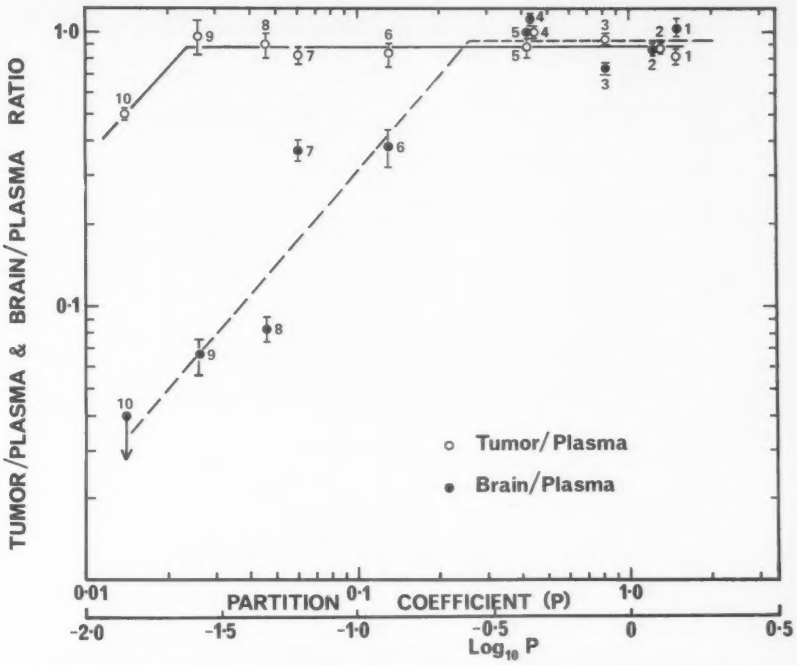


FIGURE 2.—The mean value (± 1 SE) of the tumor/plasma ratio and the brain/plasma for each of the ten 2-nitroimidazoles as a function of their octanol:water partition coefficient.

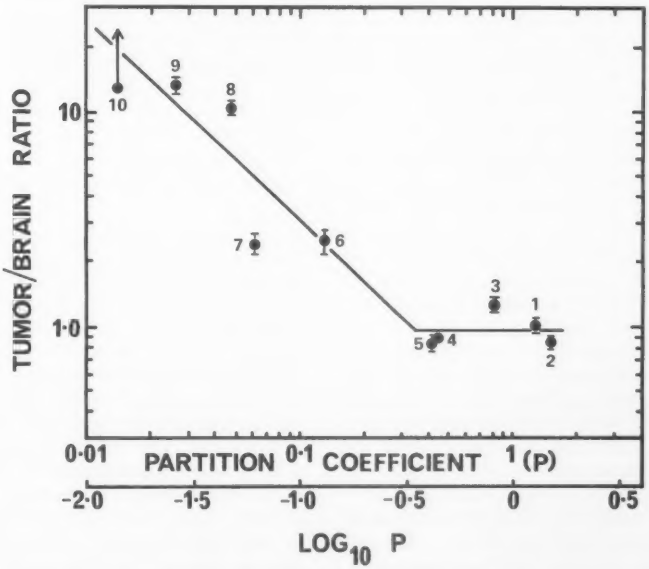


FIGURE 3.—The mean value (±1 SE) of the tumor/brain ratio for each of the ten 2-nitroimidazoles as a function of their octanol: water partition coefficient.

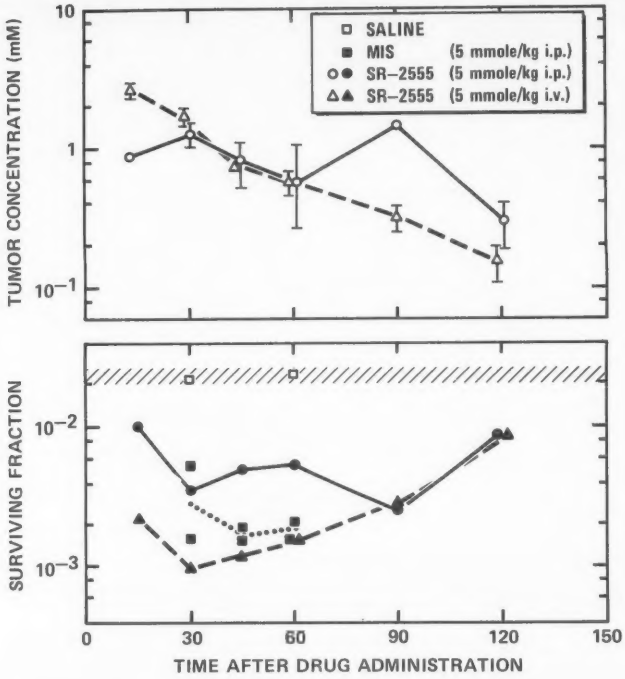


FIGURE 4.—The concentration of SR-2508 and SR-2555 in EMT6 tumors (upper panel), and the cell surviving fraction to a single dose of 2000 rad to the same tumors (lower panel) as a function of time after drug administration (in minutes). The SR-2508 or SR-2555 was injected iv at time 0. No toxicity to nonirradiated cells was found.

30 minutes after injection, SR-2530 is clearly a less efficient radiosensitizer than misonidazole as judged by this in vivo assay. The question of whether this is due to poor access to the hypoxic tumor cells or to an inability to radiosensitize despite the fact that its electron affinity is the same as misonidazole was answered by an in vitro experiment, in which it was shown to be the latter — presumably due to inadequate diffusion across the cell membrane of the hypoxic cells.

Thus we have concluded that because all 2-nitroimidazoles in the series except SR-2555 and SR-2530 radiosensitize tumors to the same extent as misonidazole, that the "optimum" value for the partition coefficient in this series must be very close to that of SR-2508. We have tested SR-2508 with seven different tumors of a variety of histological types transplanted in mice, and found that penetration is as good as that of misonidazole for all tumor types. Tumor penetration studies have also been performed with spontaneous tumors in dogs, and again SR-2508 gives peak tumor concentrations that are as good as or better than the same dose of misonidazole (21). This compound is expected to enter phase I clinical trials as a radiosensitizer in late 1981 or early 1982.

POTENTIAL OF RADIOSENSITIZERS IN CHEMOTHERAPY OR AS CHEMOTHERAPEUTIC AGENTS

Based on the fact that certain cytotoxic drugs act in some ways like radiation, it is possible that hypoxia per se might protect mammalian cells from the cytotoxic effects of certain chemotherapeutic agents. There are indications from in vitro experiments that this is the case for bleomycin (22) and actinomycin D (23).

Thus it seems reasonable to expect that the response of tumors to single doses of certain drugs would be enhanced by concomitant administration of a hypoxic cell sensitizer. There are indications that this is indeed the case. Rose and co-workers (24) have recently found that concomitant treatment of mice bearing the Lewis lung tumor with misonidazole combined with melphalan

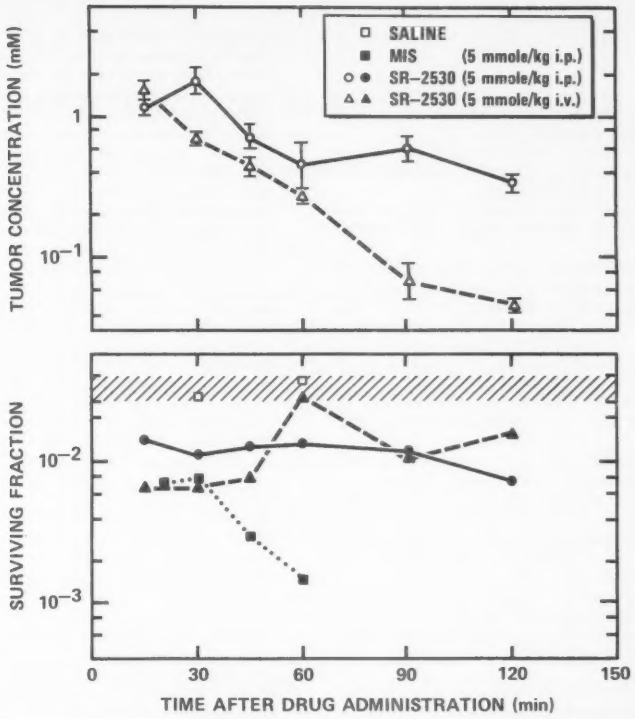


FIGURE 5.—The concentration of SR-2530 in EMT6 tumors (upper panel), and the cell surviving fraction to a single dose of 1750 rad to the same tumors (lower panel) as a function of time after drug administration. The SR-2530 was injected either ip or iv at time 0. Also shown is the surviving fraction of EMT6 tumors (done in the same experiment) to 1750 rad given at different times after a single ip injection of misonidazole at the same drug dose. No toxicity to nonirradiated cells was found.

produced a greater tumor cell kill than with melphalan alone. Moreover, the effect of misonidazole on the slope of the melphalan dose-response curve indicated true sensitization rather than additive cytotoxicity. The toxicity of the combined treatment on the bone marrow and crypt cells was less than on the tumor, showing that a real therapeutic gain had been achieved.

We have recently confirmed this result with the RIF-1-sarcoma, using regrowth delay as the endpoint after combined misonidazole and cyclophosphamide administration, and are at present extending this work to a wide range of chemotherapeutic agents. If these early results can be confirmed with a variety of tumors, which would indicate that hypoxia per se and not only distance from blood vessels or nonproliferation is a major factor protecting tumor from chemotherapeutic agents, then the potential for the hypoxic cell radiosensitizers will be far greater than originally supposed.

Finally, hypoxic cell radiosensitizers may have a future as tumor-specific cytotoxic agents because they have been shown to be toxic to cells in vitro (25,26) and to a variety of animal tumors in vivo (27,28). This is independent of the radiosensitization produced, and there is very strong evidence that it acts by an entirely different mechanism, namely by enzymatic reduction of the nitro group producing one or more toxic metabolites. Because these form only in hypoxic areas, they will be produced primarily within tumors. Although the full significance of the purely cytotoxic effects of these compounds has yet to be explored, it is already clear that they have the potential of being important tumor-specific anticancer drugs.

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Stereotactic Interstitial Irradiation for the Treatment of Brain Tumors[1]

Philip H. Gutin,[2,3,4,*] Yoshio Hosobuchi,[3] Theodore L. Phillips,[4] and Terry A. Stupar[4]

SUMMARY

Eighteen patients harboring untreated, inaccessible, slow-growing tumors or recurrent malignant primary or metastatic brain tumors were treated by the steoretactic implantation of permanent or removable radioactive seeds (^{192}Ir, ^{198}Au, ^{125}I). Seed strength was selected to deliver 10,000–12,000 rad to the tumor's periphery for ^{192}Ir, 9000–12,000 rad for ^{125}I, and 4000–7500 rad for ^{198}Au. Three of six patients implanted with ^{192}Ir seeds had objective responses for 2, 8, and 12 months, and a fourth stabilized for 20 months. Five of 9 patients treated with ^{198}Au were evaluable, three of whom responded for 3, 5, and 20 months. Two patients who received ^{125}I have been evaluated to date and are early failures. Because of its higher dose rate ^{198}Au is superior for the faster-growing malignant tumors; but because ^{125}I is easier to shield, ^{125}I seeds with greater activity are being developed for evaluation.

[Cancer Treat Rep 65 (Suppl 2):103–106, 1981]

The dose rate is the principal factor that determines the biological effect of a given dose of sparsely ionizing radiation such as x- or γ-rays. As the dose rate is lowered, the effect of a given dose is reduced because of the ongoing repair of sublethal damage. In addition, if the dose rate falls low enough, irradiated cells will again undergo mitosis. These two factors can dramatically reduce the slope of a survival curve, a phenomenon that is called the dose-rate effect (1).

For solid tumors, the dose rate effect is damped; during exposure to low dose rate radiation, tumors undergo reoxygenation, which increases radiosensitivity and balances out the continual repair of sublethal damage (2). Because they exhibit a marked dose-rate effect, normal tissues are spared much of the biological consequence of low dose rate radiation (3,4).

Radioactive interstitial or intracavitary implants characteristically irradiate tumors and limited volumes of surrounding normal tissues at dose rates of 10–100 rad/hour. Because the inverse square law of radiation applies, delivery of radiation to normal tissues is limited by the rapid drop in exposure at any incremental distance from the radioactive source (5). The ability to localize radiation from such implants and the disparity between the effects of low dose rate radiation on tumors and normal tissues makes the therapeutic ratio for brachytherapy far higher than that for conventional multifractionated teletherapy (6).

Brachytherapy has been applied extensively for palliative and curative treatment of malignancies at many sites. However, experience with brain tumors is limited and has been gained at a few, predominantly European centers. Obviously, the relative inaccessibility of brain tumors has limited work with interstitial radiation, but using stereotactic techniques Mundinger, Talairach, and others have successfully implanted radioactive gold-198 (^{198}Au), yttrium-90, and iridium-192 (^{192}Ir) into brain tumors and found a therapeutic effect (7–9). This paper describes our experience with the stereotactic implantation of radioactive seeds into the brain tumors of 18 patients.

MATERIALS AND METHODS

The patients treated harbored brain tumors less than 6 cm in diameter with one of the following characteristics: malignant neuroectodermal tumors that recurred following surgery, whole brain irradiation, and treatment with all feasible chemotherapeutic agents. Some patients could not tolerate additional chemotherapy

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because of compromised bone marrow reserves, brain metastases (arising in patients who had received previous brain radiation), or deep low-grade astrocytomas inaccessible to surgical resection. Interstitial radiation was used as primary therapy in combination with needle biopsy or as therapy for recurrence following failure of radiation therapy.

Implantation was done in two separate stereotactic procedures under local anesthesia. At the first operation, the Leksell stereotactic frame was attached and a nonradioactive (dummy) seed was placed through a burr hole to a location as near as possible to the tumor's center, using the computerized tomographic (CT) scan as a rough guide. Following this procedure, a localizing CT scan related the dummy seed to the tumor target(s) (determined by the tumor's geometry and the isotope's physical characteristics). In a separate procedure performed several days later, the radioactive seed(s) could be perfectly placed at the target(s) using the dummy seed as a reference.

Most of the radioactive seeds were permanently implanted, but some patients who received ^{198}Au had seeds mounted in catheters for later removal. This afforded greater control over dosimetry and the ability to remove the hazardous isotope to protect the surgeon should emergency decompressive surgery be required, or to protect the family when the patient was discharged from the hospital.

Patients were evaluated by neurological examinations, CT scans, and radionuclide scans at intervals of 8 weeks, when possible, and graded using the criteria developed by Levin et al (10). Patients were evaluable if they were alive and available for their first re-evaluation 8 weeks after implantation of the radioactive seed. Response was defined as a definite improvement in at least two of the three criteria in the same evaluation period if steroid dosages were stable or decreasing. Progression was defined as deterioration in two of the three criteria if steroid doses were stable or increasing. Stable disease was judged when all three tests showed no change or questionable change or when only one test showed a definite change. Time to progression was measured from the day of implantation of the radioactive seed until progression was determined as defined above. Patients receiving ^{192}Ir did not have radionuclide brain scans because of the inability of the camera to filter the gamma radiation from this isotope. This group's responses were based on two criteria only (CT scan and neurological exam).

RESULTS

Of 18 patients who received interstitial radioactive implants, 17 had previous radiation therapy, and 10 of these had previous chemotherapy. Six were treated with ^{192}Ir to a dose of 10,000–12,000 rad to the periphery of the tumor, and four had ^{125}I implants that delivered 9000–12,000 rad. Eight patients were treated with ^{198}Au to a dose of 4000–8000 rad. The only complication was a minor surgical wound infection at the site of burr hole placement in a single patient.

Three patients in the ^{198}Au-treated group were not evaluable. One refused to return for re-evaluation, one died with meningeal carcinomatosis (lung carcinoma), and one died of an overwhelming fungus infection.

Three patients reponsded to ^{192}Ir for 12-, 8-, and 2-month progression-free intervals, two others were stable for 2 and 24 months, and one patient showed deterioration at his first re-evaluation. The longest responses occurred in patients with deep low grade tumors, one of which was in transition to malignancy. All others in this group harbored a glioblastoma multiforme or anaplastic astrocytoma.

Of the five evaluable patients treated with ^{198}Au, three (one metastatic sarcoma, two glioblastoma multiformes) responded for 3, 5, and 20+ months (fig 1). One patient with a brain metastasis from systemic malignant melanoma was stable 6 months after therapy with interstitial ^{198}Au before dying of abdominal metastases. One patient with an anaplastic astrocytoma showed continuing disease progression after treatment.

Three of the patients who had ^{125}I implants had anaplastic astrocytomas, two showing progression by 8 weeks after implantation, and the third showing progression at 16 weeks. The fourth patient who received ^{125}I had a suprasellar ependymoma and remains stable at 5+ months.

DISCUSSION

After extensive experience with the brachytherapy of cancer, Pierquin (6) asked why a therapeutic dose of radiation at low dose rates over several days spared normal tissue while the same total dose given at high dose rates must be divided into multiple fractions given over several weeks to avoid normal tissue necrosis. Radiobiologists are now beginning to understand this phenomenon. The differential capacity of tumor cells and normal cells for the repair of sublethal radiation damage seems to be important, but tumor cell reoxygenation and cell cycle redistribution under continuous low dose rate radiation exposure may also be factors (1–4).

Because radiation therapy has been the most effective modality against malignant brain tumors (11) and because necrosis of normal brain has prevented delivery of more than 6000–7000 rad at conventional dose rates (12), brachytherapy is a logical and attractive treatment for brain tumors. The ability to irradiate the tumor along with only a limited volume of surrounding brain and the increased therapeutic ratio

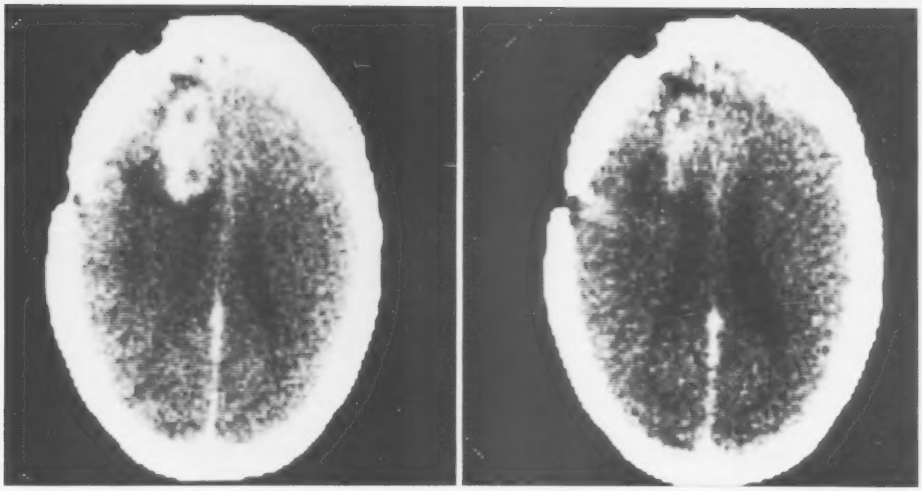

FIGURE 1.—Computerized tomographic scans (axial) showing the left frontal glioblastoma multiforme that recurred in a 50-year-old woman after surgery, radiation therapy, and chemotherapy. The tumor is shown before (left) and 2 months after (right) the stereotactic implantation of ^{198}Au for an additional 6000 rad. The nonradioactive (dummy) seed is visible (right). The patient's hemiparesis and dysphasia improved markedly for 5 months.

allow the delivery of higher initial radiation doses and permit reirradiation of tumors that recur after irradiation of the whole brain. We hope to begin adjuvant trials in the near future in which patients will have whole brain irradiation and an implant "boost" after surgical resection.

For glioblastoma multiforme, anaplastic astrocytoma, and the faster-growing brain metastases, ^{198}Au seems to be the superior isotope. The dose rate from this isotope as used here was approximately 60 rad/hour to the tumor's periphery. In comparison, ^{125}I delivered 7–10 rad/hour and failed to control rapidly progressing tumors of three patients. The critical dose rate below which tumor cells begin to divide again, even under continuous irradiation, is probably dependent on the cell cycle time for the individual tissue (13); it is not unexpected that the faster-growing tumors require higher dose rates for control. However, because ^{125}I is less energetic than ^{198}Au or ^{192}Ir by a factor of 10, it is easier to protect medical personnel and the patient's family when ^{125}I is used. In addition, there is some suspicion that ^{125}I has a relative biological effectiveness greater than unity (14). For these reasons, we have asked that ^{125}I seeds with higher specific activity (and consequently higher dose rate) be developed for testing in malignant brain tumors.

Continued refinements should increase the effectiveness of stereotactic interstitial radiation. We will soon start testing a stereotactic frame that fits into the CT scanner (15) and provides more accurate placement of isotopes and consequently more precise dosimetry. This will eliminate one surgical procedure. There is now experimental evidence that hypoxic cell sensitizers may be of value in low dose rate therapy despite the purportedly lower oxygen enhancement ratio at low dose rates[5]. Certainly, therapeutic trials of the nitromidazole sensitizers in clinical brachytherapy are practical at significantly higher drug doses than are possible with conventional fractionated radiation therapy.

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Polyamines: Relation to Brain Tumor Therapy and Monitoring[1]

Laurence J. Marton[2,3,*]

The involvement in cellular growth processes of the polyamines spermidine and spermine and their diamine precursor putrescine has been studied extensively in the last decade. The known aspects of polyamine metabolism and function in a variety of biological systems have been well reviewed (1–5).

Increased intracellular polyamine concentrations have been linked to increased rates of cell proliferation and division (1–5), and higher intracellular concentrations have been found in neoplastic cells than in normal cells. This relationship has recently been demonstrated for putrescine in human brain tumors (6). This information suggests at least two intriguing questions: (*a*) Are the polyamines in brain tumor cells released into extracellular fluids, and if so, can assaying for these compounds in biofluids such as cerebrospinal fluid (CSF) be useful for diagnosing and monitoring brain tumors? (*b*) Do compounds that interfere with polyamine biosynthesis interfere with tumor growth, and if so, can these compounds be used in therapy?

INHIBITORS OF POLYAMINE BIOSYNTHESIS

Let us look at the second question first. A number of compounds have been synthesized that interfere with polyamine biosynthesis, primarily by blocking the action of the enzyme ornithine decarboxylase (ODC), which catalyzes the formation of putrescine from ornithine, or the enzyme S-adenosylmethionine decarboxylase, which is involved both in the formation of spermidine from putrescine and of spermine from spermidine. A comprehensive review of polyamine inhibitors has been published and should be consulted for information beyond the scope of this brief report (7).

One compound, α-difluoromethylornithine (DFMO), is particularly interesting. It was synthesized by Metcalf et al (8) and is an enzyme-activated irreversible inactivator of ODC. We have begun to study the effect of this compound on the growth of 9L rat brain tumor cells in vitro. This agent is effective in inhibiting growth, without actually killing cells, and shows specificity in that the effect can be reversed by adding back the inhibited product, putrescine (9). In addition, our studies indicate that this compound augments the cytocidal effect of BCNU on 9L cells both in vitro (10) and in vivo (unpublished observations). In vivo studies conducted at the Centre de Recherche Merrell Internationale in Strasbourg (11,12) indicate activity against mouse mammary tumor with minimal to no toxicity. They have also begun preliminary human trials (12).

Obviously DFMO, or more appropriately polyamine inhibitors as a class, are compounds that deserve further study. Their potential growth inhibiting activity and their minimal toxicity suggest that they may be useful to prevent or diminish tumor regrowth in the interval between courses of presently available therapy. This aspect of brain tumor therapy has been virtually neglected, though it deserves considerable thought and effort. In addition, our results demonstrating a benefit from combining DFMO and BCNU suggest the possible use of this compound as a biological response modifier.

POLYAMINES AS TUMOR MARKERS

Now let us return to our first question of the utility of CSF polyamines as tumor markers. General screening for brain tumors based on any single marker has little hope of success, as our recent review of CSF markers points out (13). CSF cannot be obtained easily, and even if a test could be based on serum or urine, the low prevalence of brain tumors in the general population and the imperfect sensitivity and specificity of known assays produce an unacceptable number of false positive results for a single test to be practical. Multiple marker tests may alleviate some problems of sensitivity, but they too may be impractical. Thus attention has been focused on other possible uses of markers such as aiding the diagnosis of patients suspected of harboring tumor, long-term monitoring of patients with known tumor, or short-term monitoring of the efficacy of a therapeutic modality. To date,

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however, definitive data are available for the long-term monitoring of only one tumor, medulloblastoma.

As previously mentioned, brain tumors contain higher levels of putrescine than normal brain tissue (6). Because the central nervous system, including CSF, is relatively isolated from the rest of the body, intracellular fluctuations in brain tumor polyamine levels might be reflected in the CSF polyamine levels. Increased CSF polyamine levels might be due to either rapid growth or necrosis of the tumor.

Our studies to date reveal one fully defined and several potential uses for CSF polyamine determinations. Compared to a reference group of healthy adult volunteers and patients with a variety of non-neoplastic neurological diseases, patients with glioblastoma multiforme and anaplastic astrocytoma had elevated levels of CSF polyamines, and patients with tumor recurrence had levels significantly higher than stable patients (14). These studies were complicated by the occurrence of many false positive and false negative results. Elevations did not appear to precede tumor recurrence and did not augment or improve upon other available and routine diagnostic tests. We could not correlate polyamine levels with the degree of malignancy, tumor volume, or volume of tumor central low density. Only the proximity of the tumor to the ventricular system seemed to be of any consequence. Thus it seems safe to say that CSF polyamine determinations will add little to our present diagnostic capabilities for monitoring tumor progression in these patients. However, because tumors adjacent to the ventricular system showed significant elevations, these tumors together with brain stem and spinal cord tumors, which are also adjacent to CSF pathways, must be studied in greater detail.

For patients harboring medulloblastoma (15,16), we found an excellent correlation between patient status and CSF polyamine levels (putrescine in particular), and an elevation in CSF putrescine was frequently the earliest indicator of tumor recurrence. Several false negative results have been obtained, but have not and probably will not lead to inappropriate therapy. More importantly, no false positive results have been found; elevations due to other causes such as infection or vascular disease have caused no diagnostic problem. All patients who had elevated levels have had recurrences. Thus we feel that CSF polyamine determination should be a routine procedure for long-term monitoring of patients harboring medulloblastoma.

Although polyamines may reflect growth activity or the lack of it for a variety of brain tumors, the fact that these compounds cannot reach the CSF may be limiting. Studies of putrescine capillary permeability in rat brain and diffusion in cat brain have substantiated this (17). Therefore, polyamine levels appear to be a poor diagnostic tool for hemispheric gliomas that are distant from CSF pathways, but are a useful assay for medulloblastoma and other tumors adjacent to CSF pathways.

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Historical Perspective and Overview of the Third Conference on Brain Tumor Therapy[1]

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When the First Asilomar Conference on brain tumor therapy was held in November 1975, participants shared a common optimism in the future success of brain tumor chemotherapy that was based on a faith in the straightforward application of somewhat simplistic interpretations of the oncologic dogma of the late 1960s and early 1970s. Conferees represented many disciplines: neurosurgery, neurology, neuropathology, human oncology, experimental rodent oncology, radiation oncology, laboratory medicine, and biophysics—we barely spoke the same language. Over the years, most Conference participants retained and expanded their interest and work in brain tumor research and stimulated the interest of a wider group of medical scientists and clinicians in this problem. But even though this interest led to more clinical trials and to sophisticated experimentation, definable progress, measured as greater and more consistent control of tumor growth in patients, has not been realized.

Our best current clinical chemotherapeutic or multimodality therapies for the treatment of primary malignant brain tumors are palliative only. For chemotherapy adjuvant to surgery and irradiation, the benefit over irradiation and surgery is on the order of 2–3 months in 50% of patients; chemotherapy administered at recurrence can be expected, at the very best, to prevent further tumor progression for 6 months in 50% of patients (1,2).[3]

These results persist in spite of our ability to make early diagnosis of many tumors because of sophisticated neuroradiologic techniques such as computerized tomography and the use of CSF polyamines levels (particularly putrescine) to follow patients with tumors that are adjacent to the subarachnoid space or ventricular CSF pathways (eg, medulloblastomas, ependymomas, and carcinomatosis). CSF polyamine levels are elevated with tumor growth and lowered during remission, often antedating progression by months (3).

The most progress in clinical treatment appears to be the result of the use of radiosensitizers and interstitial irradiation. The use of radiosensitizers to selectively enhance the effects of radiation in hypoxic tumor regions has theoretical merit, although misonidazole, the drug currently undergoing clinical investigation, probably is not the best radiation sensitizer because of its serious dose-limiting neurotoxicity. Other agents are being developed or investigated (4). Another approach to improve the therapeutic index of radiation therapy is localized tumor irradiation with temporarily implanted radioisotopes (5). With this technique, very high radiation dose levels can be delivered rapidly and safely to a circumscribed tumor region. Because CNS tumors are not always spheres or ellipsoids and are characterized by centripedally migrating tumor cells that infiltrate "normal" brain tissue, the value of the technique may be limited. Interstitial irradiation used to expose all tumor cells would produce a margin of normal brain necrosis. In some brain regions this will be acceptable; in others, the risk to vital cognitive or vegetative functions may be high.

Adjuvant use of these techniques is possible. For instance, CNS tumor patients could receive radiation sensitizing drugs during combined interstitial irradiation and external photon irradiation. The practicality of such an approach invites clinical trial, particularly in view of the limited chemotherapeutic options available.

Clearly, our current chemotherapeutic protocols and these new techniques will not cure brain tumors. Different approaches are needed to selectively kill glial tumor cells without damage to normal glia or other normal tissue cells.

The failure of therapy is the direct result of a lack of selectivity and specificity of treatment modalities. This Conference addressed in depth the well-defined problem of pharmacokinetics of drug delivery to CNS tumors. Two other areas, clonal responsivity to therapy and heterogeneity of human glial tumors, both of which are critical determinants of the specificity of therapy, were discussed at length but not in a definitive manner for the obvious reason that little is known about differences between neoplastic and

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[3]Only papers from the Conference are cited here; each article should be consulted for a comprehensive reference list.

normal glial cells or about differences among neoplastic glial cells within a tumor.

It is clear that there is marked variation in blood flow and permeability among tumor types and within single tumors (6,7). These pharmacokinetic factors, in addition to specific in situ biological stability and physical characteristics of a drug, mediate the delivery of "adequate" therapeutic drug levels to cells at varying distances from capillaries. In order to improve tumor cell kill, tumor capillary drug levels must be increased (8). A two- to fourfold advantage in tumor drug concentration and/or integrated drug exposure ($C \times T$ or C_T) can be attained by intra-arterial infusion for many drugs for tumors that are located within the field of perfusion of an artery (eg, the middle cerebral artery) (9).

Another approach, selective arterial catheterization and injection of hypertonic solutions, produces a transient "osmotic opening" of the blood-brain barrier (< 25 minutes) and a greater than fivefold increase in brain capillary permeability by "breaking" the tight junctions between adjacent endothelial cells (9). While methotrexate (MTX) levels in dog brain can be increased 8-to 12-fold 1 hour after administration of mannitol and MTX, these levels fall approximately 100-fold in the next 6 hours (10). There is some doubt, however, that this technique can be used effectively to increase the therapeutic index of chemotherapy for CNS tumors. But the possibility that this technique might produce localized increased permeability in tumors with compromised circulation deserves further investigation.

In addition to the heterogeneity of drug delivery and its attendant therapeutic problems, heterogeneity of tumor cells among and within tumors must be intensively investigated. Nonglial tumors have been shown to have cell sublines that can be cloned for their preference to colonize different metastatic sites (11). These clones also show differing responses to in situ and in vitro chemosensitivity. Some of the clonal specificity in metastatic potential may relate to variability in membrane glycoproteins, while the variable chemosensitivity may reflect intracellular adaptation that is the result of restricted intracellular transport. gene amplification, repair of nucleic acid damage, or expansion of metabolic salvage pathways.

Over the years histopathologic criteria have been used to distinguish among glial tumors. There are many other distinguishing and contrasting features that can help to characterize glial tumor heterogeneity. Some of these are in vitro morphology, HLA phenotype, modal chromosome number and chromosome stability, enzyme activity, intracellular proteins, in vitro growth requirements, and chemosensitivity both in vitro (eg, efficiency of colony formation; CFE) and in heterotransplants in nude mice (12–16).

Even for "sensitive" tumors, the inevitable development of drug resistance is a therapy-limiting problem (17). If one accepts the premise either that resistant subpopulations of cells exist at the time of initial treatment (which is likely) or that they develop by later mutation, changes in therapy must be initiated at the approximate nadir of tumor cell burden to avoid repopulation by resistant cells that negate therapy. Unfortunately, we are not able to use different chemotherapeutic agents in sequence to suppress tumor cell repopulation because we lack effective agents. For instance, the disappointing clinical response to BCNU seems to correlate well with the limited efficacy of BCNU against human tumor cells grown in culture (14) and in nude mice (13). If these data obtained from cultured or heterotransplanted human tumor cells can be accepted at face value, BCNU achieves at best no more than a 1 to 2 log cell kill at doses equivalent to those attainable clinically.

Most drugs currently available nonspecifically alkylate biomolecules, block nucleic acid synthesis, intercalate, or interfere with metabolism of tumor cells and vital normal cells. Alternate approaches to the design of drugs will require greater insight into and understanding of tumor cell biology. One novel approach presented at the Conference was the use of irreversible inhibitors of ornithine decarboxylase to block polyamine synthesis to potentiate the effects of anticancer drugs and slow tumor growth without significant systemic toxicity to the host. While these approaches represent fresh approaches to drug development, the expectation that one drug will be found to cure cancer does not seem well-founded.

The consensus of conferees was that there is no simple solution to effective control of malignant brain tumors. If selectivity and specificity of tumor cell kill are to be achieved, new drugs and new approaches to drug design prerequisite to tumor control will require a much better understanding of glial cellular processes that can be obtained by innovative hard work and stable funding for research.

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